

**INVESTIGATIONS INTO THE MECHANISM OF ACTION
AND ANTIFUNGAL ACTIVITY OF THE BOTANICAL
COUMARIN SURANGIN B**

by

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ABSTRACT

The mechanism of action of surangin B, a coumarin from the roots of *Mammea longifolia*, was examined in bovine heart mitochondria and mouse brain synaptosomal preparations. The potential antifungal activity of surangin B was also investigated.

Complexes II, III and IV of bovine heart mitochondria were strongly inhibited by surangin B, but complex I was insensitive to this compound. Analysis of surangin B's inhibition of complex II used submitochondrial particles. Surangin B displayed non-competitive kinetics when either succinate or decylubiquinone were used as substrates, indicating that it binds to a site which is distinct from both the succinate binding site and the domain responsible for interacting with ubiquinone. Difference spectra of reduced complex III equilibrated with surangin B closely paralleled those of antimycin A, but were very different to those of the selective Q_o site inhibitors myxothiazol and famoxadone. Other experiments used the electron acceptor 2-nitrosofluorene, which intercepts electrons specifically from the Q_i site. These experiments confirmed that like antimycin A, surangin B selectively blocks electron diversion to 2-nitrosofluorene through Q_i within complex III.

Surangin B causes presynaptic release of both neurotransmitter and non-neurotransmitter amino acids from mouse brain synaptosomes. The stimulatory effect of surangin B and other complex III-specific inhibitors on amino acid release was inhibited by N,N,N',N'-tetramethyl-*p*-phenylenediamine suggesting that complex III blockade in mitochondria of the nerve ending is an important mechanism causing release.

Surangin B also inhibited *in vitro* mycelial growth and spore germination in several species of fungi. As an inhibitor of mycelial growth surangin B showed strongest activity against *Rhizoctonia solani* ($IC_{50} = 3.8 \mu M$) and *Botrytis cinerea* ($IC_{50} = 11.2 \mu M$), but inhibitory effects were less pronounced in *Alternaria dauci*, *Fusarium oxysporum* and *Penicillium sp.* ($IC_{50}s > 30 \mu M$) and absent in *Trichoderma harzianum*. Surangin B reduced the level of spore germination in *Fusarium oxysporum* ($IC_{50} = 2.3 \mu M$) and *Botrytis cinerea* ($IC_{50} = 1.4 \mu M$), although *Alternaria dauci* was considerably more tolerant of this coumarin ($IC_{50} = 500 \mu M$). The activity of surangin B compared favorably with certain commercial fungicides indicating that coumarins of this type may have potential as antifungal agents.

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LIST OF ABBREVIATIONS

AA	antimycin A
AAF	2-acetylaminofluorene
ACh	acetylcholine
ALA	alanine
Asp	aspartic acid
ATP	adenosine triphosphate
ADP	adenosine diphosphate
CB	carboxin
CCCP	carbonylcyanide <i>m</i> -chlorophenylhydrazone
CN	cyanide
CO	carbon monoxide
Complex I	NADH dehydrogenase
Complex II	succinate-CoQ oxidoreductase
Complex III	ubiquinone-cytochrome c oxidoreductase
Complex IV	cytochrome oxidase
CoQ	ubiquinone or coenzyme Q
CTRL	control
DB	2,3-dimethoxy-5-methyl-6- <i>n</i> -decyl-1,4-benzoquinone
DMSO	dimethylsulfoxide
DCIP	2,6-dichlorophenolindophenol
DDT	dichlorodiphenyltrichloroethane
Dith	dithionite
DMF	dimethylformamide
EC ₅₀	concentration that is effective in producing a 50% change in response
EDTA	ethylene diamine tetraacetic acid
EGTA	ethyleneglycol- <i>bis</i> -(β -aminoethylether) N,N,N',N'-tetraacetic acid
EPSC	excitatory postsynaptic currents

ETC	electron transport chain
FAD	flavin adenine dinucleotide
FCCP	carbonylcyanide- <i>p</i> -(trifluoromethoxy)-phenylhydrazone
F	famoxadone
FMN	flavin mononucleotide
GABA	γ -aminobutyric acid
GLU	glutamic acid
HQNO	heptylhydroxyquinoline- <i>N</i> -oxide
HPLC	high performance liquid chromatography
5- HT	serotonin
ISP	iron sulfur protein
IC ₅₀	concentration required to caused 50% inhibition
MOA	β -methoxyacrylate stilbene
MPP ⁺	1-methyl-4-phenylpyridine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MX	myxothiazol
2-NOF	2-nitrosofluorene
NADH	nicotinamide adenine dinucleotide
NE	norepinephrine
NEM	n-ethylmaleimide
N-OH-AF	N-hydroxy-2-aminofluorene
NP	nitropropionic acid
NSF	NEM sensitive fusion protein
O-CB	oxycarboxin
OD	optical density
OPA	<i>o</i> -phthalaldehyde
OSCP	oligomycin sensitivity-conferring protein
Q _i site	ubiquinone-reducing site
Q _o site	ubiquinol-oxidizing site
Rot	rotenone
SB	surangin B

SER	serine
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulphate
SMPs	submitochondrial particles
SNAP	NSF-attachment protein
SNARE	soluble NEM-sensitive-factor attachment protein receptor
TAU	taurine
TCA cycle	tricarboxylic acid cycle
TMPD	N,N,N,N',N'-tetramethyl- <i>p</i> -phenylenediamine dihydrochloride
TTFA	thenoyltrifluoroacetone
TTX	tetrodotoxin
UQ-ol	ubiquinol
VTD	veratridine

CHAPTER 1. INTRODUCTION

1.1 Botanical Insecticides – an Overview

Botanical insecticides are conveniently defined as phytochemicals having the capacity to kill or severely impair the function of insects. Naturally occurring plant toxins were first recognized as insect controlling agents by indigenous cultures long before they were exploited by modern societies. By the late 1800's, about half a dozen botanical insecticides were in common use in Europe and North America and this situation continued until the introduction of the first synthetic organic insecticides in the early 1940's. Of these, only the pyrethrins and rotenone are currently utilized in significant quantities. The pyrethrins continue to be used to great advantage in the protection of grain and natural fibres in storage and farm animals from arthropod (primarily insect) attack, as well as in the control of household and disease-transmitting pests (mainly flies and mosquitoes). Rotenone is applied principally in the control of animal ectoparasites, garden arthropod pests and is also employed as a piscicide. Low level use of ryania and sabadilla still occurs to protect some organically grown crops from insect attack (Zang et al, 1998), whereas nicotine is rarely used as a botanical insecticide, having been discontinued in the early 1990's in many countries (Duke, 1990).

Predictably, those natural product insecticides which specifically interfere with nerve or muscle function are not generally noted for additional pesticidal activities. But substances, which for example target mitochondria, may have the capacity to disrupt function in arthropods, plants and fungi and precedents for broad spectrum pesticidal

activity of compounds with this mode of action are known. For instance, the synthetic dinitrophenols have been used commercially as insecticides, fungicides and herbicides (The Pesticide Manual, 1977). Similarly, the mitochondrial inhibitor and natural product insecticide rotenone has also found a niche as a piscicide, although it does not possess useful herbicidal or fungicidal properties. In Chapter 4 of this thesis, evidence is presented to support the hypothesis that the potent mitochondrial inhibitor and insecticide surangin B also has inherent antifungal activity.

Approximately half the species of organisms that inhabit planet earth are insects. Of these, about 10,000 impact negatively on humankind and can therefore be classified as pests (Metcalf and Metcalf, 1993). Insects are recognized as a major pest of agricultural and forest products, since they damage about one-third of the world's food and fiber crops, worth billions of dollars, each year (Jacobsen, 1989). Natural plant-driven toxins can be of clear benefit in managing pest populations, especially within an integrated pest management framework. In some instances natural toxins from plants can also provide useful leads to the discovery and development of synthetic products that are superior to the natural product in terms of effectiveness against pests and safety to many non-target organisms. A landmark achievement in this regard was the discovery of the synthetic pyrethroids which emerged from pyrethrin prototypes (Naumann, 1990; Chuman et al., 2000). The potential for development of more potent synthetic insecticidal analogs from other botanicals, for example, the *N*-isobutylamides is well-recognized (Blade, 1989).

In addition to classical botanical insecticides, considerable research has also been carried out on semiochemicals such as host recognition chemicals and antifeedants (Pickett et al., 1991; Pickett et al., 1988; Ley et al., 1993). The natural plant material

neem targets behavioural and endocrine processes in insects and a number of neem-containing products are now registered in North America and Europe (Ascher, 1992). Despite some successes, it is clear that botanical insect control agents command a very small proportion (approximately 1 %) of the total insecticide market. The first part of the 21st century will therefore continue to emphasize a heavy reliance on synthetic chemicals such as the pyrethroids and neonicotinoids for insect control (Elliott, 1995; Maienfisch et al., 2001; Maienfisch et al., 2004).

The remaining discussion of Chapter 1 aims to provide an overview of the more important botanical insecticides in use today and includes the rotenoids, veratrum alkaloids, pyrethrins and neem as well as those which have potential in insect control such as the *N*-isobutylamides and insecticidal coumarins. The chapter then leads into a review of mitochondrial function and the modes of action of agents which interfere selectively with mitochondrial function. This is followed by a overview of the nerve and neurotransmitter release. I end Chapter 1 by laying out my research objectives.

Rotenoids

Rotenone and its congeners (the rotenoids) are phytochemicals with useful arthropodicidal and piscicidal properties (Haley, 1978; Schnick, 1974). Rotenoids are found in over sixty species of leguminous plants where they occur at significant concentrations in the roots, bark and fruits (Perry et al., 1998). Commercial production of rotenoids is now mainly limited to *Derris eliptica* which is cultivated in Indonesia, Malaya and the Philippines, and to various members of the *Lonchocarpus* genus which are grown in South America and are extracted to provide cube resin. Anecdotal reports on

the application of rotenoid-containing plant parts to waters by indigenous peoples to intoxicate fish, and facilitate their collection for food, go back at least two hundred years (Sharp, 1961; Santi and Toth, 1965). As botanical arthropodicides, the rotenoids have been used to control agricultural pests since the 1840s (Negherbohn, 1959; Fukami and Nakajima, 1971, Perry et al. 1998). The chemical structure of rotenone (the major pesticidally active principle of the rotenoids; see Fig 1.1) was reported in 1933 (LaForge et al., 1933) and its total synthesis was announced some 30 years later (Miyano, 1965).

Rotenone formulations continue to be used by fisheries biologists to control introduced piscine species identified as a hazard to aquatic ecosystem integrity (California Department of Fish and Game, 1997). The acceptability of this and other pesticidal uses of rotenone is facilitated by the compound's ease of photodecomposition and chemical breakdown, often within days of application (Cheng et al., 1972), although in winter the duration of activity can extend to several months (Schnick 1974). Rotenone is extensively metabolized by microbes (Sariaslani et al., 1984), mammals, insects and fish (Fukami et al., 1971). In animals, several sites on the molecule are susceptible to oxidation by cytochrome P₄₅₀ monooxygenases, giving rise to a variety of less toxic water soluble products that can be excreted (Fukami et al., 1969). Rotenone's high acute toxicity towards arthropods and fish can be attributed to the relatively low levels of cytochrome P₄₅₀ present in these organisms (Fukami et al., 1969). The interaction of rotenone with NADH:ubiquinone oxidoreductase (complex I) of mitochondria is considered the primary mechanism (Singer and Ramsay, 1994) leading to acute toxicity in mammals, insects and fish (Fukami, 1976; Hollingworth and Ahammadsahib, 1995; Ueno et al., 1994). Rotenone has no effect on complexes II-IV since the oxidation of

succinate or ascorbate in the presence of tetramethyl phenylene diamine is unaffected (Lindahl and Oberg, 1960). In this context rotenone represents an excellent biochemical tool for achieving selective inhibition of complex I.

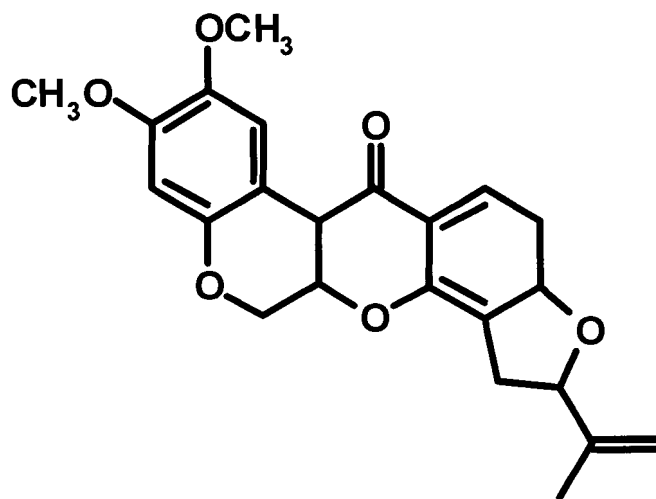


Figure 1.1 The structure of rotenone.

There is mounting evidence that rotenoids have anticancer potential, since it is known that rotenone reduces the frequency of liver and mammary tumors in rodents when administered in the diet (Hansen et al., 1965; Cunningham et al., 1995) and also blocks proliferation of hepatocytes *in vitro* (Cunningham et al., 1995). Furthermore, inhibition of phorbol ester-induced ornithine decarboxylase activity, which provides a useful index of cancer chemoprevention activity, is observed with deguelin and related rotenoids (Luyengi et al. 1994; Gerhauser et al., 1996). More recent studies using human

breast cancer cells confirm that inhibition of induced ornithine decarboxylase activity by rotenoids and also pyridaben and fenazaquin (structurally unrelated miticides) occurs as a consequence of their inhibition of NADH:ubiquinone oxidoreductase (Fang and Casida, 1998; Rowlands and Casida, 1998).

Clearly, NADH:ubiquinone reductase may represent a useful therapeutic target for development of cancer chemoprevention strategies, however, it is also known that during sustained exposure of rodents to rotenone, brain NADH:ubiquinone reductase is blocked and this results in typical Parkinsonian symptomology including hyperkinesia, rigidity and degenerative changes to nigrostriatal dopaminergic tracts (Betarbet et al., 2000; Alam and Schmidt, 2002). Similar effects been reported for the protoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Tipton and Singer, 1993; Singer and Ramsay, 1990), heightening concern that substances that inhibit NADH:ubiquinone reductase can also play a role in the development of Parkinson's disease in humans.

Veratrum Alkaloids (Sabadilla)

Plants of the Liliaceae family, in particular the North American species (*Veratrum viride* Aiton), its closely related European counterpart (*Veratrum album* L.) and a Central American species *Veratrum sabadilla* (*Schoenocaulon officinale* Gray) have long been known to contain substances useful in the control of ectoparasites of humans and domesticated animals (Griffiths, 1847; Maisch, 1885; Matthyse and Schwardt, 1943). The bioactive components are mainly associated with the roots of *Veratrum viride* and *Veratrum album* and the seeds of *Veratrum sabadilla* and can be extracted with organic solvents as a complex mixture of lipophilic alkaloids commonly known as veratrine or

sabadilla. Of significant interest has been the broad spectrum insecticidal activity and blood pressure lowering effects of the crude alkaloid extract (Ikawa et al., 1945; Wintersteiner, 1953). Sabadilla continues to provide a control option in organic farming (Zang et al., 1997). The two most active alkaloids in sabadilla, the ester alkaloids veratridine and cevadine (Fig 1.2), interfere with the function of voltage-gated sodium channels (Ohta et al., 1973), an action which accounts for both their insecticidal and hypotensive effects.

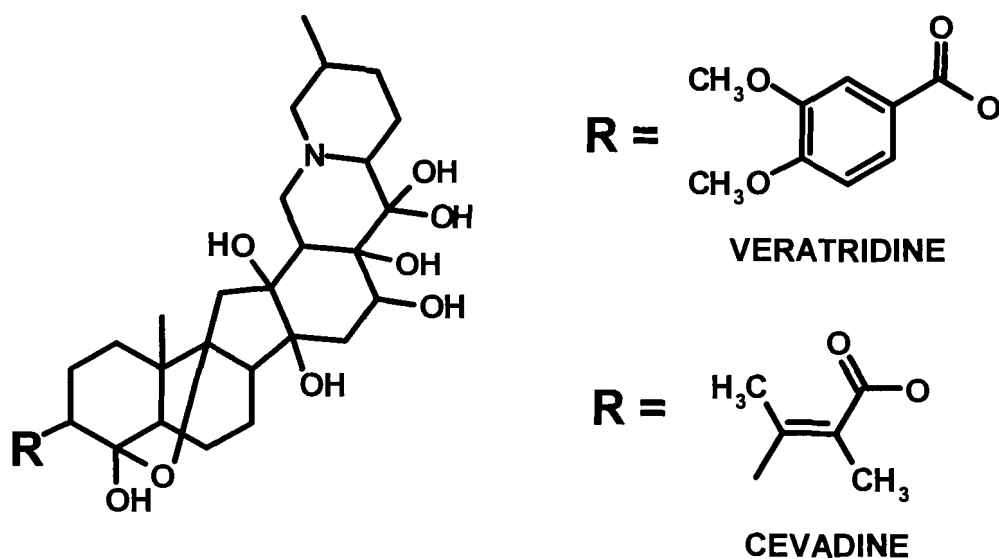


Figure 1.2 The structures of veratridine and cevadine.

The majority of mechanistic investigations have focused on veratridine which causes sustained opening of voltage-gated sodium channels at resting membrane

potentials (Ulbricht, 1998). The inward movement of sodium ions results in a depolarization of the nerve membrane (Ohta et al., 1973), which leads to increased neuronal sodium-potassium pump activity and in presynaptic nerve terminals, secondary calcium influx via activation of voltage-gated calcium channels (Satoh and Nakazato, 1991; Zhang and Nicholson, 1993), which activate neurotransmitter release. Veratridine and other alkaloid neurotoxins (batrachotoxin, grayanotoxin and aconitine) interact selectively with neurotoxin binding site 2 on voltage-gated sodium channels (Catterall, 1980) and are assumed to access this binding region through the lipid phase of the membrane (Ulbricht, 1998). A number of allosteric interactions have been demonstrated between the alkaloid binding site (site 2) and sites 3, 5, 7 and 9 on the sodium channel which bind respectively, β -scorpion venoms (Izhar et al., 2004), the marine phycotoxins, brevetoxin and ciguatoxin (Van Dolah et al., 1994), insecticidal activators (DDT and pyrethroids) and therapeutic drugs such as local anesthetics, anticonvulsants and antiarrhythmics (Strichartz, 1976; Dullenkopf et al., 2003; Zimanyi et al., 1989; Kendig, 1981; Ragsdale et al., 1991; Wang and Wang, 2003). Similar allosteric interactions between site 2 and the less well-characterized binding sites on sodium channels including those for dihydropyrazoles (Deecher et al., 1991), *N*-alkylamides (Ottea et al., 1989) and anandamide (Nicholson et al., 2003) have also been reported.

More recently Wang and Wang (1998) discovered that point mutations in segment I-S6 of voltage-gated Na^+ channels cause the channel to become functionally resistant to batrachotoxin. Total insensitivity to an extremely high concentration (5 mM) of batrachotoxin was observed with an Asn434Lys mutation, whereas the Asn434Ala mutation showed some sensitivity to this alkaloid. Moreover, channels possessing the

Asn434Lys mutation showed virtually normal current kinetics after exposure to veratridine with very slight slowing of the tail current. Veratridine does however inhibit the peak Na^+ current in this mutant form suggesting that batrachotoxin and veratridine dock with site 2 binding region in subtly different ways.

N-Isobutylamides

Biologically active *N*-isobutylamides (also known as lipid amides and *N*-alkylamides) occur naturally in plants of the Compositae, Piperaceae and Rutaceae families (Su, 1985) and vary widely in the acid moiety (see Figure 1.3).

N-isobutylamides are noted both for their insecticidal activity, which can approach that of the pyrethrins (Metcalf, 1955), and also for their ability to elicit intense tingling and local anesthetic effects on the tongue and lips (Jacobson, 1954a). These effects however do not always parallel each other. References can also be found in the literature to vasodilatory, antioxidant, molluscicidal and insect growth inhibitory properties of *N*-isobutylamides (Miyakado et al., 1989; Sumitomo Chemical Company Patent 212 150; Kubo et al., 1984).

Affinin-containing plant extracts were found to be topically active to a variety of dipterous, lepidopterous, coleopterous and hemipterous insects (Su, 1985) and numerous other naturally occurring *N*-alkylamides have been investigated as potential insect control agents (Jacobson, 1971; Bohlmann et al. 1973). More recently a range of *N*-alkylamide analogs have been synthesized with improved insecticidal properties (Elliott et al., 1987a; Elliott et al., 1987b; Miyakado, 1982; Crombie and Denman, 1984; Wellcome Foundation Patent, 1984). Despite intensive effort, none of these synthetic compounds

have demonstrated sufficient stability or activity in the field to warrant commercial development.

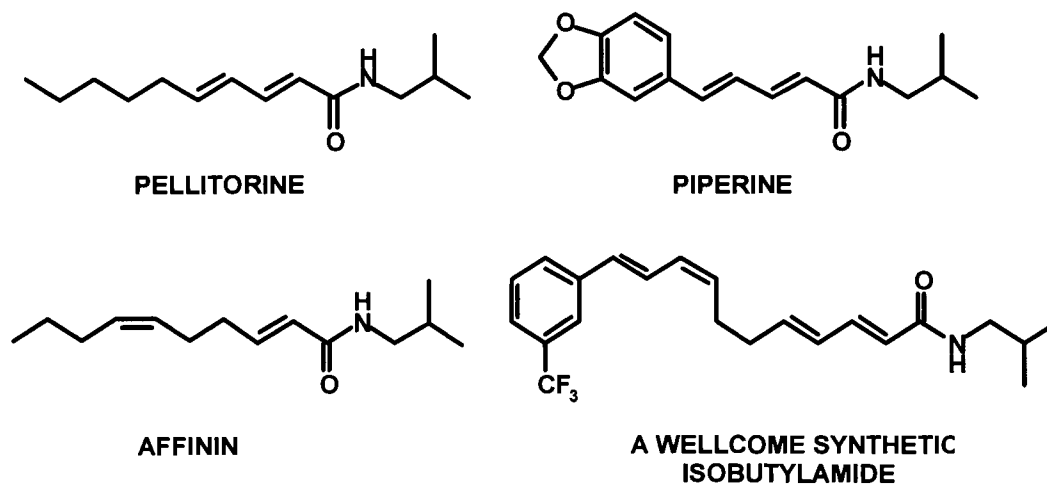


Figure 1.3 Structures of natural and synthetic isobutylamides.

A neurotoxic action of isobutylamides similar to pyrethroids was demonstrated in insects and biochemical studies found that tetrodotoxin blocks isobutylamide-induced release of the neurotransmitter γ -aminobutyric acid (GABA) from mammalian synaptosomes suggesting that these compounds activate sodium channels (Burt et al., 1984). Subsequent investigations showed isobutylamides produce multiple spiking and suppression of nerve conduction in housefly nervous system, block sodium currents and extend tail current decay times in locust neuronal somata (Blade et al., 1989; Lees and Burt, 1988). Moreover, veratridine (sodium channel)-dependent release of acetylcholine

from cockroach synaptosomes is blocked when they have been previously incubated with isobutylamides (Nicholson et al., 1985). Consistent with these findings, Ottea et al. (1989) described inhibition or stimulation of ^{22}Na uptake by the N-isobutylamide BTG 502 in mouse brain synaptoneurosomal fractions depending on the type of sodium channel activation, and blockade of [^3H]batrachotoxinin A-20- α -benzoate binding.

In addition to insecticidal effects arising through targeting of sodium channels, isobutylamides of *Echinacea* species have been found to inhibit the biotransformation of arachidonic acid to prostaglandins which accounts for certain anti-inflammatory actions of echinacea (Jacobson, 1967; Bauer and Remiger, 1989). The anti-inflammatory potency of echinacea preparations can be standardized by quantitating isobutylamide content (Bauer, 1997) or approximated by gauging the intensity of the tingling or numbing sensation after application to the tongue. Isobutylamides from *Echinacea* are known to be insecticidally active (Jacobson, 1954b).

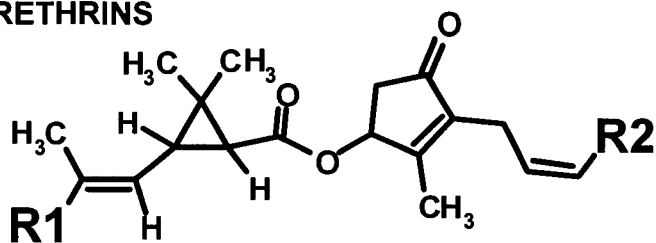
Pyrethrins

The pyrethrins are the insecticidal components of pyrethrum oleoresin, obtained from solvent extraction of the flower heads of the pyrethrum plant, *Tanacetum cinerariaefolium*, a member of the chrysanthemum family. Pyrethrum plants are grown commercially in Kenya as well as several other African countries and agricultural production also occurs in Tasmania (Gullickson, 1995). The term pyrethrins describes a total of six esters based on coupling of chrysanthemic acid or pyrethric acid to a cyclopentenolone alcohol moiety (Crombie 1995; Fig 1.4 a). Pyrethrin I and pyrethrin II account for the majority of the kill and knockdown activity respectively (Hendrick, 1994).

Pyrethrins I and II are also the most abundant compounds in pyrethrum oleoresin, amounting to 55 – 70 % by weight (Head, 1973). Pyrethrum extract has a low order of toxicity to warm-blooded species (Gray and Soderlund, 1985). Although pyrethrins are lethal to a broad range of insect species (Elliott and Janes, 1973), all six esters break down rapidly when exposed to air, moisture or light, causing loss of insecticidal activity (Alan and Miller, 1990). This inherent instability ensures extremely short environmental persistence (Crosby, 1995), a property that limits pyrethrin use mostly to control of arthropod pests of domestic, medical and veterinary importance (Gerberg, 1995; Kennedy and Hamilton, 1995).

Even so, an enormously successful expansion into agricultural insect control has been made possible with the pyrethroids, synthetic analogs of pyrethrins (Naumann, 1990), which have been designed to provide greater residuality during insect control operations while retaining the original botanical ester attributes of potent insecticidal activity and relatively low mammalian toxicity (Elliott, 1995; Schoenig, 1995).

a) THE PYRETHRINS



	R1	R2	%
PYRETHRIN I	CH₃	CH₂ = CH₂	35
CINERIN I	CH₃	CH₃	10
JASMOLIN I	CH₃	CH₂ - CH₃	5
PYRETHRIN II	CH₃ - O - CO -	CH = CH₂	32
CINERIN II	CH₃ - O - CO -	CH₃	14
JASMOLIN II	CH₃ - O - CO -	CH₂ - CH₃	4

b) ALLETHRIN

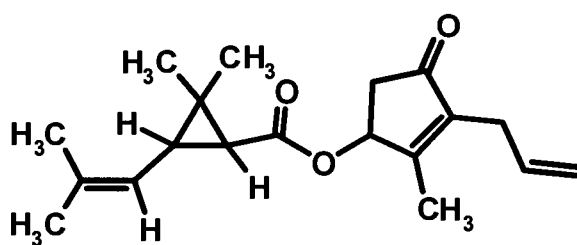


Figure 1.4 Structures of the pyrethrins (a) and allethrin (b).

The rapid knockdown and lethal actions of pyrethrins and pyrethroids in arthropods occur as a result of these chemicals rapidly accessing the nervous system and causing functional impairment. Amongst the first experiments on mode of action of pyrethrins were those of Lowenstein (1942) who discovered that pyrethrum extract causes multiple spiking followed by nerve block in the giant fibre pathway of the cockroach (*Blatta orientalis*). Cuticular application of pyrethrins was later shown to produce multiple spiking in the crural nerve of the American cockroach (*Periplaneta americana*) (LaLonde and Brown, 1954). More recently the studies of Burt and Goodchild (1971) showed that topical application of pyrethrin I (the most insecticidal botanical ester from *Tanacetum cinerariaefolium*) to the American cockroach increases spiking activity in the sixth abdominal ganglion and eventually blocks action potentials in giant fibre axons, effects that parallel the progression of poisoning symptoms. Although subsequent experiments on chrysanthemic acid esters mostly focused on pyrethroids, the investigations with allethrin (a very close structural analog of pyrethrin I; Fig. 1.4 b) are widely considered to provide information that is relevant to the neurotoxic actions of the botanical esters.

The voltage clamp technique has given important insights into the ionic basis for the excitatory actions of allethrin on nerve (Narahashi and Anderson, 1967; Murayama et al. 1972; Vijverberg et al, 1982). Under voltage clamp conditions the sodium channels of nerves poisoned with allethrin show almost normal voltage-dependence of opening. However, when sodium channels are activated by step depolarization, allethrin delays their closing, leading to a slowly-decaying current (known as the "tail current") upon repolarization (Fig. 1.5).

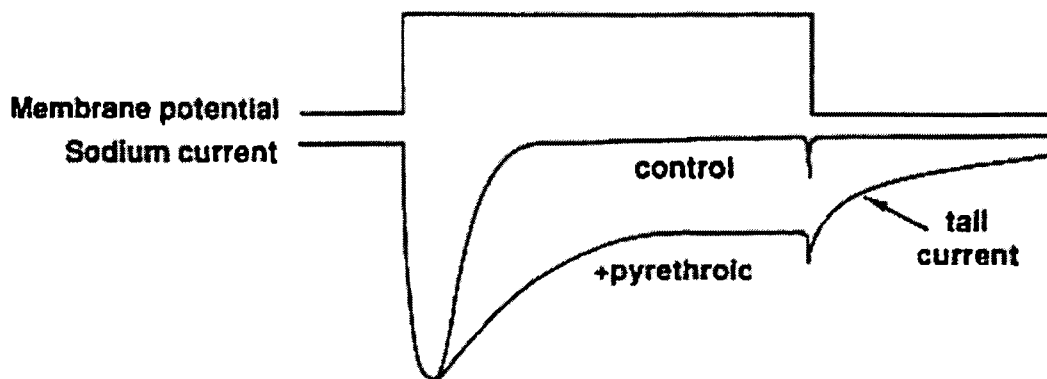


Figure 1.5 Sodium channel tail current as described by Soderlund, (1995).

This mechanism explains the multiple spiking and negative after potential observed during intracellular recording of allethrin's action in cockroach giant axons (Narahashi, 1962). Taken together these experiments identify the voltage-gated sodium channel as the primary site of action for allethrin and by analogy, pyrethrin I. The eventual block of electrical activity observed by Lowenstein (1942) and Burt and Goodchild (1971) likely arises from bioenergetic failure of the nerve as a result of the persistent excitation and multiple spiking caused by the botanical esters. Although the sodium channel is the only target implicated in the neurotoxic action of pyrethrin I in insects to date, it must be remembered that certain synthetic pyrethroids have affinities for potassium (Narahashi and Anderson, 1967), calcium (Brooks and Clark, 1987;

Symington et al., 1999; Duce et al., 1999) and chloride channels (Bloomquist et al., 1986; Burr and Ray, 2004) and the ability of pyrethrin I to act these targets is unclear.

Azadirachtin

The neem tree, *Azadirachta indica* of the Meliaceae family was originally native to India but is now widely distributed in many tropical and subtropical countries. Over 60 triterpenoids together with numerous other constituents have been isolated from extracts of the trunk, bark, leaves, roots, fruit and seeds of this tree (Jones et al., 1989). Most prominent amongst these botanicals in terms of potential for insect control is azadirachtin (Warthen 1989) which is present at highest concentrations in the seeds (Butterworth and Morgan, 1986) and in common with some other neem components can potentially interfere with feeding, reproduction and development in a range of insects, as well as promote deterency (Schmutterer, 1990; Koul and Isman, 1991; Isman et al., 1991; Lee et al., 1991; Verkerk and Wright, 1993).

Evidence in support of azadirachtin's ability to disrupt feeding and growth comes from various studies. In Mexican bean beetle, 1-10 ppm azadirachtin typically suppresses feeding and weight gain and after several days larvae and other life stages succumb to starvation (Butterworth and Morgan, 1986; Rembold, 1988; Isman et al., 1990). The primary antifeedant effect can involve either activation of deterrent sensilla or suppression of stimulating sensilla in the maxillary region (Mordue and Blackwell, 1993), although neem bioactives may also prevent insects from converting food into biomass (Martinez and van Emden, 1999).

Various reproductive effects of azadirachtin have been reported including degenerative changes to trophic cells of ovarioles in the Mexican bean beetle and suppression of ovarian follicle growth in the migratory locust (Schmutterer, 1990). Moreover, at low doses of azadirachtin, male milkweed bugs were unable to copulate and fruit flies lost the ability to respond to male pheromone (Schmutterer, 1988).

In 1990, Schmutterer also proposed that azadirachtin interferes with developmental programming in insects by disrupting hormonal systems, particularly those involving the ecdysteroid ecdysone. Azadirachtin causes dose- and time- related effects on ecdysis and death can take place before or during molting (Mordue and Blackwell, 1993). Azadirachtin also depletes juvenile hormone levels in insects possibly by preventing its synthesis and secretion from the corpora cardiaca in insect brain (Garcia and Rembold, 1984; Garcia et al., 1990; Mordue and Blackwell, 1993).

Repellency is likely mediated by an olfactory response since it occurs in the absence of direct contact with neem-treated plants. Such effects have been reported in the brown rice planthopper and cabbage webworm (Schmutterer, 1990) and likely involve volatile sulfur-containing organic compounds of neem (Balandrin et al, 1988), rather than azadirachtin itself.

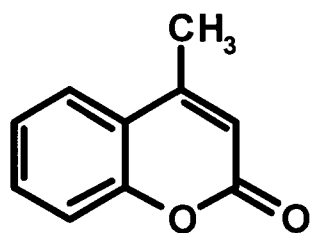
Limitations in the successful control of insect pests in the field with azadirachtin-containing extracts have been encountered due to the low chemical stability of this triterpenoid and its slow action in altering behavior and various other physiological processes in target insects. However, its toxicity towards certain parasitoids and some predators of pests is relatively low indicating potential usefulness in integrated insect pest management programs (Schmutterer, 1988). Another advantage of azadirachtin is its

virtual lack of toxicity towards warm-blooded animals, in fact, neem has been used by humans for centuries for various therapeutic and contraceptive purposes (Koul et al, 1990).

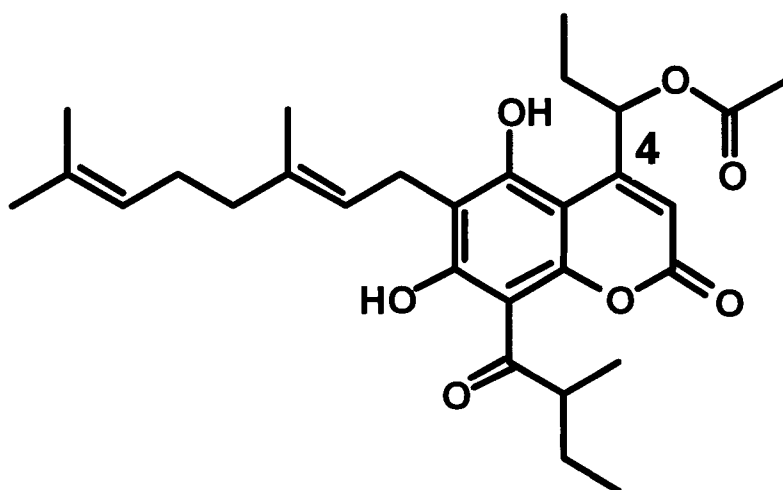
Insecticidal Coumarins

A considerable number of chemically diverse coumarins are found as secondary metabolites in green plants and as metabolic products in bacteria and fungi (Murray, 1989; Murray, 1991; Hoult and Paya, 1996). From the structural perspective, coumarins consist of a bicyclic system made up of a fused α -pyrone and benzene ring (Fig 1.6 a). Many substitution patterns are possible on the central bicyclic system giving rise to a variety of biological effects and various pharmacological and potential therapeutic properties have been attributed to natural product coumarins (Ko et al., 1989; Teng et al., 1992a; Teng et al., 1992b; Huang et al., 1992). Extracts of *Mammea americana*, an evergreen tree of the Caribbean region, for example, shows toxicity to a variety of insects (Plank, 1944; Morris and Pagan, 1953) and coumarins were subsequently identified as the active principles. According to Morton (1987), an awareness of the insecticidal properties of *Mammea americana* dates back at least to 1864 when Grosourdy published a report in *El Medico Botanico Criollo* describing the toxic effects of certain parts of this tree. When seeds of *Mammea americana* were imported into the U.S.A. in 1919 from Ecuador, the record compiled by the United States Department of Agriculture noted potential insecticidal and medicinal uses. As far as insecticidal activity in *Mammea americana* is concerned the seeds are by far the most insecticidal parts of the tree and studies by the Federal Experimental Station in Puerto Rico have found seed extracts to be active against

armyworms, melonworms, cockroaches, ants, termites, adult and larval stages of mosquito, flies, diamond-back moth and aphids in various oral and contact assays (Morton, 1987). Moreover, concentrated aqueous extracts of seeds or fruits were effective at killing fleas and ticks on dogs. Interestingly the dogs did not display signs of poisoning but larger scale studies in mice resulted in 6 % mortality. Extracts of *Mammea americana* seeds are also acutely toxic to fish but 50 to 100 times less potent than derris. Anecdotal evidence suggests that the seeds also cause poisoning in birds and hogs. In her book, Julia Morton (Morton, 1987) draws attention to the observation that although fruits of *Mammea americana* have "formed part of the diet of the inhabitants of the Caribbean for many generations, it is well known that this fruit produces discomfort, especially in the digestive system in some persons". Morton and colleagues also noted "reports of poisoning in humans are known" and compared the fruit of *Mammea americana* to those of akee (*Blighia sapida*) which are also toxic.



(a) Structure of coumarin



(b) Structure of surangin B (note the 4-(1-acetoxypropyl) side chain)

Figure 1.6 Chemical structure of coumarin and surangin B.

Various investigations, which have been conducted on the insecticidal dust and organosoluble extracts of mature seeds of *Mammea americana*, have been reviewed by Crombie (1990). Up until the late 1960s, a number of coumarins had been isolated from *Mammea americana* and their structures successfully determined, however, they all failed to explain the insecticidal activity of the crude extracts. A few years later Crombie et al. (1972) identified a coumarin possessing a 4-(1-acetoxypentyl) side chain which accounted for much of the insecticidal activity of this species. A 4-(1-acetoxypentyl) coumarin, surangin B (Fig. 1.6 b), had also been identified in *Mammea longifolia*, a related species indigenous to Madagascar and the Western Ghats of India and was shown to have bactericidal properties (Joshi et al. 1969). Later toxicity assays on mosquito larvae, houseflies and crickets confirmed that surangin B is also insecticidal (Crombie et al. 1972; Nicholson and Zhang, 1995), and generally more potent as an insecticide than its counterpart from *Mammea americana* (Crombie et al. 1972).

The lack of rapid knockdown of insects dosed with surangin B accompanied by a progressively deepening paralysis, parallels the symptomology of certain compounds which target mitochondria (Nicholson and Zhang, 1995). Experiments in our laboratory using insect muscle mitochondria confirmed surangin B to be a potent blocker of state 3 respiration when driven by NAD^+ - or FAD^+ -linked substrates, but that it has no effect on mitochondrial complexes I and IV, indicating that complexes II or III may be sensitive to inhibition by this coumarin (Zheng et al. 1998). Surangin B was further found to mimic cyanide in reducing whole body levels of ATP when applied to insects *in vivo* (Zheng et al. 1998). In common with rotenone, surangin B also inhibits the utilization of oxygen by mouse brain synaptosomes and depolarizes intrasynaptosomal mitochondria (Nicholson

and Zhang, 1995). Other experiments revealed that this coumarin stimulates both release of tritium label from [³H]choline-loaded insect synaptosomes (Zheng et al. 1998) and release of preloaded [³H]GABA from mammalian synaptosomes (Nicholson and Zhang 1995). These results indicate that mitochondrial blockade leading to bioenergetic failure in muscle and nerve are major mechanisms in the development of paralysis in insects exposed to surangin B. Further mechanistic investigation of the action of surangin B in muscle and nerve represents a significant part of this thesis.

1.2 The Mitochondrion as a Site of Neurotoxicant Action

Mitochondria are amongst the largest of the cytoplasmic organelles and represent a critical site of metabolite and energy interconversion. Mitochondria can be found in virtually all eukaryotic cells and these structures represent the main location of cellular ATP synthesis under aerobic situations (Margulis and Lynn, 1981).

There are six distinct mechanisms by which toxicants interfere with mitochondrial function. Firstly, respiratory chain inhibitors such as rotenone, carboxin, antimycin, and cyanide block electron flow at complexes I to IV, respectively, and in consequence inhibit substrate-driven mitochondrial oxygen consumption in the presence of either adenosine diphosphate (ADP) or uncouplers. Secondly, phosphorylation inhibitors such as oligomycin suppress the ADP-dependent burst of oxygen consumption but fail to block uncoupler-stimulated respiration. Thirdly, uncoupling agents including dinitrophenol, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) abolish the efficient coupling between electron flow down the respiratory chain and the phosphorylation of ADP, which is a key

characteristic of physiologically intact mitochondria. In contrast, certain transport inhibitors including atractyloside and bongrekic acid block the export of ATP from mitochondria, or the import of molecules across the mitochondrial inner membrane, processes essential to the bioenergetic cycle. Next, certain ionophores especially valinomycin and nigericin render the inner membrane permeable to ionic species leading to disruption of compartmentalization. Lastly a number of Krebs cycle inhibitors are known such as arsenite, fluorocitrate and aminooxyacetate which block one or more of the Krebs cycle enzymes or an ancillary reactions, thus reducing formation of NADH or FADH₂, which provide a vital shuttle of reducing equivalents to the electron transport chain.

For the purposes of dissecting the functional properties of mitochondria, the three most important classes of mitochondrial toxicants have been electron transport inhibitors, uncouplers and phosphorylation inhibitors. A large number of compounds, including many pesticides, have now been found to interfere selectively with discrete targets in mitochondria and modify function. Thus a variety of valuable pharmacological probes are available for use in exploring the mechanism of action of novel mitochondrial toxicants, such as surangin B.

1.2.1 Structure and Morphology of Mitochondria

Mitochondria can vary in size considerably, but as a general statement they are typically 1.0 - 4.0 μm in length and approximately 0.4 - 1.0 μm diameter. Mitochondria frequently have an elongated appearance when observed under the electron microscope,

and when examined in living cells some degree of alignment of their long axes can often be seen. The number of mitochondria in a given cell is known to vary with cell type and mitochondrial numbers often correlate with the metabolic requirements of the cell (Scheffler, 1999; Sherratt, 1991). Mitochondria are also mobile within the cytoplasmic compartment and actively divide to produce further mitochondria.

The mitochondrion consists of two membranous envelopes, the outer and inner membranes, and each consists of a phospholipid bilayer with a unique assembly of integral proteins (Daum, 1985). These membranes divide the mitochondrion into two internal compartments, the intermembrane space and the central matrix. The outer membrane is a smooth continuous envelope that forms the surface of the mitochondrion or the outer mitochondrial boundary. This membrane has a very high phospholipid to protein ratio, close to 0.9 (Daum, 1985). The proteins associated with the outer membrane include enzymes of fatty acid synthesis, nucleotide diphosphokinase and porin (De Pinto et al., 1987). Porin is a channel-forming protein which allows ions and molecules with a molecular weight less than 5,000 daltons to traverse the outer membrane, but restricts the passage of larger proteins and other macromolecules (Parsons, 1965; Mannella, 1982).

The inner membrane forms the boundary separating the two mitochondrial compartments, the intermembrane space and the matrix (Sherratt, 1991). The inner membrane is highly convoluted and folds extensively forming cristae that protrude into the matrix provide an appropriate surface area for energy yielding reactions of the mitochondrion. The number of cristae and their morphology thus reflect an adaptation of mitochondria to the energetic demands of the cell. For example, extensively folded

cristae with large surface areas are consistently observed in muscle and neuronal cells since respiratory rates can reach very high levels. The inner membrane has a relatively low phospholipid to protein ratio, of about 0.3 (Daum, 1985) and is also very impermeable with transfer of most solutes across the membrane requiring a specific transporter (Li et al., 1990). Also, embedded in the inner membrane is the electron transport chain which is made up of five discrete membrane protein complexes, NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome *c* reductase (complex III), cytochrome *c* oxidase (complex IV) and the ATP synthase which is also known as complex V (Hatefi et al., 1975).

As already described, there are two mitochondrial compartments. The intermembrane space is a rather narrow region between the inner and outer membranes and it is normally assumed to be a continuation of the cytosol with its own enzymes. In fact, the composition of small molecules and ions in the intermembrane space closely resembles that of the cytosol since small molecules are free to pass through the porin channels in the outer membrane (De Pinto et al., 1987). The mitochondrial matrix is the compartment enclosed by the inner membrane and therefore represents the central core compartment of the mitochondria. The matrix has a high protein content and contains mitochondrial DNA and ribosomes (Nobrega and Tzagoloff, 1980), the former coding for some, but not all, of the mitochondrial proteins.

1.2.2 The Physiological Function of Mitochondria

Mitochondria perform a range of critical cellular functions. Under aerobic situations their primary function is to utilize the energy derived from oxidation of organic

molecules such as glucose to synthesize ATP, the main energy currency molecule of the cell. In addition to generating ATP by oxidative phosphorylation, mitochondria synthesize lipids (Scheffler et al., 1999), heme (Meyer and Schmid, 1973), amino acids (Beattie et al., 1970) and also represent a key organelle in the regulation of intracellular pH and ion homeostasis (Simpson, 1967; Rasmussen, 1971).

The inner mitochondrial membrane performs a variety of critical bioenergetic functions. Most obvious of these is to establish and conserve the H^+ electrochemical gradient and use it to drive the phosphorylation of ADP. The inner membrane contains the essential components of the respiratory chain including NADH dehydrogenase, succinate dehydrogenase, iron-sulfur proteins (ISP), cytochromes *b*, *c*₁, *c*, *a* and *a*₃ together with the ATPase energy transduction complex and its associated components and coupling factors, such as *F*₁ and the oligomycin sensitivity-conferring protein (OSCP) (Nalin and Cross, 1982). Most proteins can only gain access to the matrix if they are selectively transported through the inner membrane, a process which requires a specific transporter and serves to tightly regulate the spectrum of proteins residing in the matrix.

1.2.3 Linkage between Glycolysis and Tricarboxylic Acid Cycle

The majority of organisms are exposed to oxygen and cells in an aerobic environment readily convert glucose to pyruvate via the glycolytic pathway, or glycolysis (Passarella and Quagliariello, 1976). These glycolytic reactions take place in the cytoplasmic compartment of the cell and yield two ATP molecules per molecule of glucose by substrate level phosphorylation and also two molecules of nicotinamide adenine dinucleotide (NADH). Pyruvate diffuses rapidly through the outer mitochondrial

membrane and after transportation across the inner membrane it undergoes reductive decarboxylation to acetyl coenzyme A (CoA), the substrate for the TCA cycle (Passarella and Quagliarello, 1976; Halestrap, 1978). Acetyl-CoA is then fed into the cyclic pathway of the TCA cycle (Tyler, 1992; Metzler, 1977), which is the series of reactions that oxidizes the carbon skeleton of acetyl-CoA to carbon dioxide and generates a further two ATP molecules per molecule of glucose by substrate level phosphorylation. The enzymes that carry out these reactions are located in the mitochondrial matrix and at the matrix surface of the inner membrane (Metzler, 1977). However, by far the most important bioenergetic products of the TCA cycle are NADH and flavine adenine dinucleotide (FADH₂), which transfer the high energy electrons gained from acetyl-CoA oxidation to the electron transport chain. Oxidation of one molecule of acetyl-CoA in the TCA cycle eventually generates 22 molecules of ATP via the electron transport chain and chemiosmosis.

1.2.4 Electron Transport, Oxidative Phosphorylation and Energy Production

From a bioenergetic perspective, the most important function of mitochondria is to carry out respiratory or oxidative phosphorylation. This is a multistage process by which the energy stored in NADH and FADH₂ is used to produce ATP. It consists of two functional parts, the electron transport (or respiratory chain) and ATP synthesis.

The electron transport chain (ETC) is present in the cristae of the inner mitochondrial membrane (Gilkerson et al., 2003). There are two main pathways for the passage of electrons. The first pathway is from complex I (where electrons from NADH are accepted) to complex III and then on to complex IV. The second route allows

electrons from FADH_2 to be accepted by complex II and then passed through complex III to complex IV. (Fig. 1.7) These two pathways of electron flow converge at ubiquinone [or coenzyme Q (CoQ)], which serves as a mobile electron transfer molecule linking complex I and II with complex III. The respiratory chain consists of these four integral membrane protein complexes (complex I – IV) and two freely-diffusible carrier molecules, ubiquinone and cytochrome *c*. Complex I oxidizes NADH and complex II oxidizes succinate (generating FADH_2) and both use ubiquinone as the electron acceptor. Ubiquinone then reduces complex III which donates electrons to cytochrome *c*. Reduced cytochrome *c* is then oxidized by complex IV and electrons finally are passed to molecular oxygen as the ultimate electron acceptor (Gupte et al., 1984).

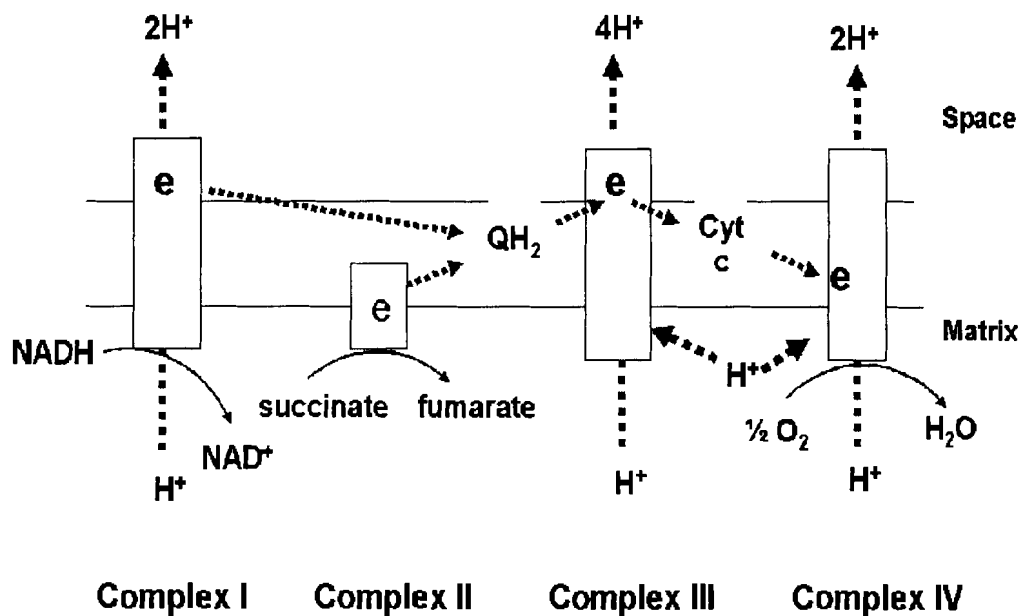


Figure 1.7 The electron transport chain. (QH_2 = ubiquinol; $\text{Cyt } c$ = cytochrome *c*; e^- = electron).

Within the electron transport chain, electrons are moved from molecules with low reduction potential (low affinity for electrons) to molecules with successively higher reduction potential (higher electron affinity). The energy released during electron transport is used to move protons. All the complexes except complex II (succinate dehydrogenase) pump protons from the matrix into the intermembrane space as they transfer reducing equivalents (either hydrogen atoms or electrons) from one carrier to the next. As a result, the energy released during electron transport can be stored as a proton gradient across the membrane. These protons are eventually channeled back into the matrix via the ATPase, which drives the synthesis of ATP as they return.

Oxidative phosphorylation traps the free energy stored in the electrochemical proton gradient across the inner mitochondria membrane and couples it to ATP synthesis. The biosynthesis of ATP is catalyzed by the enzyme ATP synthase which resides within the inner mitochondrial membrane (Schnaiman and Pedersen, 1968). The ATP synthase is a protein complex composed of three main subunits including F_0 which is embedded in the membrane (Hamasur and Glaser, 1992), F_1 which protrudes from the inside of the inner membrane into the matrix (Abrahams et al., 1994); and oligomycin-sensitive coupling protein (MacLennan and Tzagoloff, 1968; Tzagoloff et al., 1968) which connects to F_0 . ATP synthase binds ADP and inorganic phosphate at its catalytic site and requires a proton gradient for substrate coupling (Hatefi et al., 1975; Boyer, 1993). The rate of oxidative phosphorylation is closely regulated by the ratio of ADP/ATP in mitochondria. When ADP levels rise and inorganic phosphate is available, proton flow through ATP synthase is elevated and this stimulates the biosynthesis of ATP. Oligomycin is an antibiotic which binds to the ATP synthase and blocks the proton channel, so inhibiting

ATP synthesis. This means that oligomycin inhibits the synthesis of ATP by specifically interfering with oxidative phosphorylation without inhibiting the initial conservation of energy as a proton gradient, which forms the proton motive force described by Peter Mitchell (Mitchell, 1980). As mentioned already, oligomycin binds to a protein associated with F_0 (Glaser and Norling, 1983), and because it selectively affects the F_0 component of the ATP synthase oligomycin inhibits coupled respiration but fails to block uncoupled respiration.

1.2.5 Mitochondrial Ca^{++} Transport and Ca^{++} Buffering

Mitochondria from a number of vertebrate sources possess active mechanisms for the uptake and release of Ca^{++} (Nicholls and Ferguson 1992). Exposure of isolated mitochondria to Ca^{++} concentrations greater than 1 μ M, normally result in uptake of this cation from the surrounding medium into the matrix, via a uniport mechanism. The inner mitochondrial membrane also uses an independent efflux pathway, operating as a $Ca^{++}/2Na^+$ antiporter, which achieves electroneutral exchange (Brand, 1985). The Na^+ translocator component of this antiporter relies on simultaneous operation of the Na^+/H^+ exchanger (Crompton and Heid, 1978; Li et al., 1992; Rasmussen, 1990), which drives the rapid removal of Ca^{++} from the matrix.

The uptake of Ca^{++} by mitochondria also requires the presence of inorganic phosphate (P_i) which is taken up alongside Ca^{++} . In the presence of external P_i , the rising pH of the matrix causes P_i to enter the matrix via the P_i^-/H^+ symporter. In addition to neutralizing the increase in internal pH, this mechanism allows the accumulated Ca^{++} to complex with the P_i to form an osmotically inactive calcium phosphate “gel” (Weinbach

et al, 1967; Lehninger, 1970). Under conditions where cytoplasmic free Ca^{++} concentration exceeds the buffering capacity of mitochondria, a build up of Ca^{++} in the cytoplasm can often occur. This buffering capacity of mitochondria appears to have evolved in part as a protection mechanism to prevent cytoplasmic free Ca^{++} reaching a level which will damage the cell. This mitochondria mechanism can also protect the cell under pathological conditions when cytoplasmic free Ca^{++} increases (Lehninger, 1970). However studies show that mitochondria can only store Ca^{++} on a short term basis, so when normal function resumes as cytoplasmic free Ca^{++} concentration falls below the mitochondrial trigger point for uptake, the efflux pathway takes over, reducing matrix Ca^{++} which can then be extruded from the cell (Nicholls and Akerman, 1982; McCormack et al., 1990).

When neuronal voltage-gated sodium channels open, Na^+ rapidly enters the nerve and brings the membrane potential towards threshold, generating an action potential. This increase in cytoplasmic Na^+ level has been shown to stimulate Ca^{++} efflux from the matrix of brain mitochondria (Nicholls and Crompton, 1980). Also, action potentials invading the nerve ending trigger the opening of voltage-gated calcium channels, causing Ca^{++} entry into the nerve ending. Much of the Ca^{++} that enters is very rapidly taken up from the cytoplasm into the matrix of mitochondria. The accumulated Ca^{++} is then released back into the cytoplasm of the nerve ending generating the increase in concentration of cytoplasmic calcium in the terminal which triggers physiological release of neurotransmitters. Intrasynaptosomal mitochondria therefore play a significant buffering role in the regulation of cytosolic Ca^{++} , and that this in turn influences the net transport of Ca^{++} across the plasma membrane (Scott et al., 1980).

1.2.6 Complex I and its Inhibitors

Complex I (NADH-quinone oxidoreductase) is the one of three energy transducing systems of the respiratory chain of mitochondria (Horga and Singer, 1968). A large proportion of the electrons which cross the respiratory chain enter via complex I. Complex I is a large integral membrane complex with at least seven iron-sulphur clusters (Han et al., 1988). The redox center of complex I is composed of NADH dehydrogenase with flavin mononucleotide (FMN) as a cofactor (George and Ferguson, 1987), plus non-heme-iron proteins having at least one iron sulphur center (Nicholls and Ferguson, 1992; Walker, 1992; Weiss et al., 1991). The function of complex I is to transfers electrons from NADH to CoQ. The flow of electrons through complex I with its highly exergonic free energy change provides the energy to pump protons from the mitochondrial matrix into the intermembrane space thus contributing to ATP synthesis.

NADH produced in the cytosol provides the electrons for the NADH dehydrogenase of complex I and in animal mitochondria NADH is transferred into the matrix by a shuttle system. Interestingly, plant mitochondria have an external NADH dehydrogenase which can oxidize NADH and transfer reducing equivalents directly to ubiquinone or complex III (Weiss et al., 1991). In contrast to animal NADH dehydrogenase the plant enzyme is insensitive to rotenone as has also been found for many fungi.

Clearly, a large number of compounds have been discovered which interact selectively with mitochondrial complex I and the other mitochondrial complexes. However, for the purposes of my thesis, the discussion of this section and the sections

which immediately follow, will be limited mostly to those inhibitors and electron translocating probes that have assisted in the mechanistic investigation of surangin B action. Note also that the mode of action of rotenone has already been discussed in Chapter 1.1 and that MPTP is included because of its similarity of action to rotenone.

Of the inhibitors of complex I, some occur naturally and some are synthetics and these have been divided into two classes on the basis of their kinetic properties. Class I inhibitors inhibit complex I in a partially competitive manner with respect to ubiquinone, for example: piericidin A, annonin VI, phenalamid A₂, aurachins A and B, thiangazole, and fenpyroximate; class II inhibitors act in a noncompetitive manner, the best known of these is rotenone. All complex I inhibitors appear to interfere with transfer of electrons at discrete points between the high potential iron-sulphur cluster and ubiquinone (Gutman et al., 1970; Krueger et al., 1990).

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

In 1982, meperidine was illicitly manufactured and sold on the street as a heroin substitute. Many batches contained the impurity MPTP which was found to produce severe and irreversible end-stage Parkinsonian symptoms in many young users. PET scanning using 6-fluorodopa showed most severely symptomatic patients to have selective lesioning of the substantia nigra in brain. MPTP is now known to be converted into its active metabolite 1-methyl-4-phenylpyridine (MPP⁺) (Betarbet et al., 2000), which is then selectively accumulated by dopaminergic neurons via the plasma membrane dopamine transporter. Once it has been taken up into the cell, MPP⁺ accumulates in the mitochondria where it inhibits complex I. MPP⁺ appears to bind to

complex I near the rotenone binding site (Ramsay et al., 1991). Since dopaminergic neurons selectively uptake MPP^+ via the synaptic dopamine transporter, only dopaminergic neurons accumulate MPP^+ in significant quantities. This explains why the inhibition is not systemic but instead highly selective for dopaminergic neurons (Javitch et al., 1985) and leads to a Parkinsonian-like syndrome in humans (Langston et al., 1983a; Langston et al., 1983b; Spencer et al., 1987). MPP^+ also generates free radicals in isolated mitochondria, which have been implicated in the irreversible inactivation complex I (Cleeter et al., 1992). Recent studies indicate that high, systemically administered doses of the selective complex I inhibitor rotenone also produces selective degeneration of dopaminergic neurons in the striatum and induces Parkinsonian-like symptoms (Alam and Schmidt, 2002).

1.2.7 Complex II and its Inhibitors

Complex II provides a fundamental link between the TCA cycle (in the form of succinate) and the membrane-bound electron-transport system. Succinate dehydrogenase (SDH) makes up the extrinsic, water-soluble domain of complex II which contains a common active site for its substrate succinate. This enzyme has the redox flavine adenine dinucleotide (FAD) covalently bound and is associated with three iron-sulfur clusters (Tyler, 1992). SDH catalyzes the interconversion of succinate and fumarate. The catalytic site responsible for succinate oxidation is on the flavoprotein subunit (SDH1) and the covalently bound FAD functions as the electron acceptor. The flavin semiquinone and iron-sulfur centers of the iron protein (SDH2) then transfer electrons once at a time to the ubiquinone reductase site (Sherratt, 1991; Salerno, 1991) which provides reduced

ubiquinone (ubiquinol) to the mobile intramembrane pool which interfaces with complex III.

Malonate

Malonate (malonic acid) is a well-established selective inhibitor of succinate dehydrogenase. This compound is closely related in structure to succinate (see Figure 1.8). Mechanistically it operates as a reversible competitive inhibitor of succinate dehydrogenase since it binds to the succinate binding site in such a way that it can be displaced progressively by increasing concentrations of succinate (Webb, 1966; Mandrik et al., 1983). *In vivo* studies show that intrastriatal injection of malonate gives rise to dose-dependent excitotoxic lesioning in the striatum (Beal et al., 1993; Greene et al., 1993; Henshaw et al., 1994), but that little damage occurred if succinate and malonate were co-injected or if succinate was administered soon after malonate. These *in vivo* findings are broadly consistent with a reversibly acting competitive effect on brain succinate dehydrogenase.

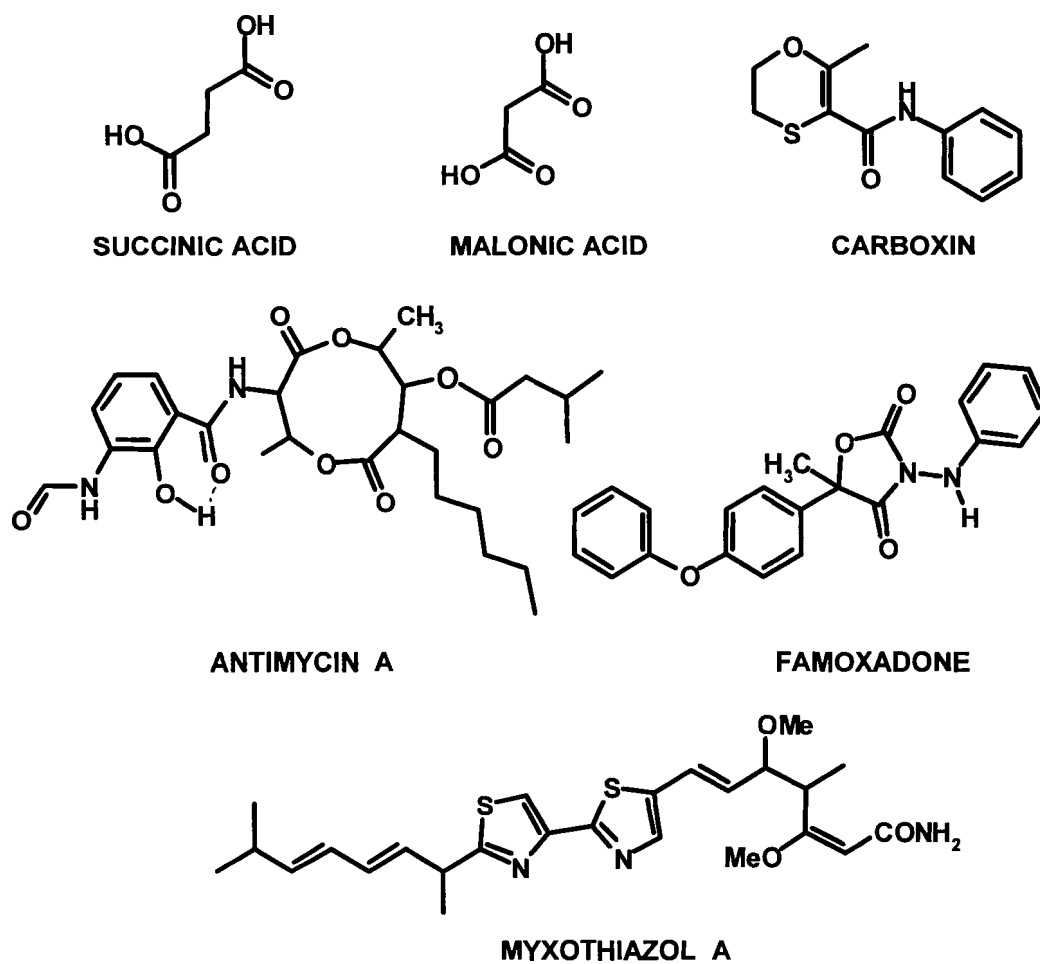


Figure 1.8 The structures of succinic acid, malonic acid, carboxin, antimycin A, famoxadone and myxothiazol A.

Carboxin

Carboxin is a systemic anilide fungicide and a selective inhibitor of complex II (White, 1971). Its structure is displayed in Figure 1.8. Carboxin acts by interfering in some way with the binding of ubiquinone to its active site. The block would be either by exclusion of ubiquinone from the active site or by occlusion of a pore that leads to the active site (Matsson et al., 2001). Which ever mechanism is operating, previous kinetic experiments with ubiquinone make it clear that carboxin affects binding of this redox intermediate to its recognition site on complex II in a non-competitive (i.e. indirect) way (Mowery et al., 1976). Inhibition of succinate dehydrogenase activity by carboxin is also predominantly non-competitive with respect to succinate (White, 1971). These latter findings of Mowery et al. and White were confirmed in my experiments which compared the inhibitory kinetics of surangin B to those of carboxin using succinate and ubiquinone as substrates. The related structural analog oxycarboxin has been shown to block complex II in a similar way (White and Thorn, 1975). Carboxin has broad spectrum antifungal properties and it is widely used as a seed treatment for control of smut, rot, and blight on barley, oats, rice, cotton, vegetables, corn and wheat. It is also used to control fairy rings on turf grass. The systemic properties of this fungicide enable it to be used to prevent both the initial establishment of fungal disease and as a therapy for fungal disease that has already started to spread within the plant (Ware, 1986).

1.2.8 Complex III and its Inhibitors

Complex III exists in the inner mitochondrial membrane as a dimer and must be surrounded by a complete annulus of phospholipid for optimum activity *in vitro*. The

complex III of bovine heart has 8 - 9 tightly bound cardiolipin molecules per monomer and their dissociation from the core complex causes irreversible loss of activity (Gomez and Robinson, 1999).

The most prominent subunits from the functional perspective are the cytochromes *b* and *c*₁, and the Rieske iron sulphur protein (ISP), since they are the only subunits participating directly both in electron transfer and the associated proton translocation (Baum et al., 1967). The cytochrome *b* subunit binds two heme groups known as *b*_L and *b*_H. *b*_L is located near the P (cytoplasmic) side of the inner mitochondrial membrane and *b*_H located close to the N (matrix) side of the membrane (Trumpower, 1990a; Trumpower, 1990b). On the basis of X-ray diffraction data to a resolution of 2.9 angstroms, atomic models of the main protein components of the bovine cytochrome bc₁ complex include core 1, core 2, cytochrome *b*, cytochrome *c*₁, and an amino-terminal fragment of the iron-sulfur protein (Iwata et al., 1998). The proteins core 1 and core 2 appear to be structurally similar to each other (Xia et al., 1997).

Complex III has two reaction centers for ubiquinone, designated Q_i and Q_o (Baum et al., 1967; Robertson et al., 1993; Tan et al., 1993). Ubiquinone functions as a mobile carrier between complexes I and II to complex III (Snyder et al., 2000). There are two properties which enable ubiquinone to act in this way. Firstly, this carrier molecule can be readily oxidized and reduced (Robertson et al., 1993). Secondly, its lipid solubility enables it to diffuse rapidly in the membrane environment, rapidly transporting hydrogens from one side of the inner membrane to the other (Baum and Rieske, 1966; Baum et al., 1967).

The Q cycle is initiated when reduced QH_2 which is generated by complex I or II on the matrix side of the membrane diffuses across the membrane and gives up two protons to the intermembrane space. The first electron liberated in this process comes from the oxidation of QH_2 to the semiquinone. This electron is passed sequentially to the Rieske iron sulphur protein of complex III, $\text{cyt } c_1$ of the complex III and then finally to cytochrome c . The second electron is liberated when the semiquinone is fully oxidized to ubiquinone (Q), and this passes to the first heme b_L , and then on to the second heme b_H . Q then diffuses back to the matrix side where it is reduced to the semiquinol by the heme b_H . During this first half of the Q cycle the net reaction is the oxidation of QH_2 to the semiquinone, the reduction of one cytochrome c and the transfer of two protons to the intermembrane space. The overall reaction is that four protons are translocated to the outside for every pair of electrons passing through complex III from QH_2 to cytochrome c (Baum et al., 1966; Silman et al., 1967; Trumpower, 1990a; Trumpower, 1990b; Ding et al., 1992; Matsuno-Ygi and hatefi, 2001).

The inhibitors of complex III can be classified to two groups according to the specific sites on this complex that they target. The Q_i or quinone reduction center is located on the matrix side of the inner membrane is associated with the recycling of half of the electrons back into the quinone pool and the uptake of protons from the matrix. The classical inhibitors of the Q_i site are 2-heptyl-4-hydroxyquinoline-n-oxide (HQNO) and antimycin A. At the Q_o center which located near the outer face of the inner membrane, electrons from reduced ubiquinone are accepted and divided into two pathways: half for recycling, and half for transfer via the iron-sulphur center and $\text{cyt } c_1$ to cytochrome c . The inhibitors of Q_o site include methoxyacrylate (MOA) stilbene (Rich et

al., 1991), myxothiazol and famoxadone (a recently commercialized agricultural fungicide).

Antimycin A

Antimycin A (see Figure 1.8 for structure) is secreted by the microbe *Streptomyces griseus* and has the ability to potently inhibit electron transfer in complex III between b_H and b_L and much less between b_L and the iron sulfur protein. It therefore is classified as a Q_i site inhibitor. In the intact respiratory chain it prevents the oxidation of both NADH and succinate, but is unable to inhibit respiration in the presence of N,N,N,N',N'-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) (Tokutake et al., 1993) the latter compound providing a means for electrons to bypass complex III completely and thus overcome the Q_i site block by antimycin. This pharmacological manipulation known as the TMPD shunt was very useful in identifying complex III as a major site of surangin B action in synaptosomes (see Chapter 3, Figure 3.10). Antimycin is known to have insecticidal activity towards the housefly and almond moth and shows a relatively wide spectrum of antifungal activity. Antimycin is also extremely toxic to fish. Antimycin has not achieved any practical significance in pest management, although proposals for this compound as a possible alternative to rotenone in fish eradication programs have been put forward.

Famoxadone

Famoxadone (see Fig. 1.8 for structure) is a member of a new class of oxazolidinone fungicides which show high activity against a variety of plant fungal

pathogens. Famoxadone combines relatively broad spectrum of activity with low mammalian toxicity and minimal environmental impact (Sternberg et al., 2001). Famoxadone is a potent inhibitor of complex III (Jordan et al., 1999a; Zheng et al., 2000; Gao et al., 2002) and achieves its inhibition by binding selectively to the Q_o reaction centre, which is the site of bifurcation of electron transport between the Rieske iron-sulfur cluster and heme b_L of complex III (Jordan et al., 1999b).

Myxothiazol

Myxothiazol is a natural product antibiotic produced by various species of *Streptomyces*. Its chemical structure, which was determined by Gerth et al. (1980) and Trowitzsch et al. (1980), incorporates the methoxyacrylate toxophore (see Fig. 1.8). Myxothiazol is highly effective against a broad range of filamentous fungi (Gerth et al., 1980), but shows negligible activity against bacteria (Thierbach and Reichenbach, 1981). Myxothiazol exerts its antifungal action by binding exclusively to the cytochrome b_L component of the Q_o site of mitochondrial complex III. Myxothiazol can therefore block the reduction of both the Rieske centre and heme b_L , although the heme b_H can still be reduced via the Q_i site via reversed electron flow using reduced hydroquinone. This mechanism accounts for the observation that 2-nitrosofluorene, which transfers electrons from the Q_i -centre directly to oxygen (Klohn et al., 1996; see Figure 2.16a), relieves myxothiazol's inhibition of complex III. I used 2-nitrosofluorene in my thesis research to help identify the binding site of surangin B on complex III (see Figure 2.16b).

The onset of the myxothiazol's inhibitory effect on complex III is rapid and, at its maximum effect concentration, oxygen consumption is almost fully blocked (Thierbach

and Reichenbach, 1981). The synthetic methoxyacrylate fungicides ICIA5504, azoxystrobin and kresoxim-methyl form a very important group of recently developed commercial fungicides based on myxothiazol and the simpler natural product methoxyacrylate strobilurin A (Clough and Godfrey, 1995; Henry and Gustafson, 2003).

1.2.9 Complex IV and its Inhibitors

Complex IV (also known as cytochrome c oxidase and cytochrome *a/a₃*) is the terminal enzyme of the electron respiratory chain (Hatefi, 1985). This complex has a molecular weight of about 200,000 Da and consists of 13 discrete polypeptides arranged as two catalytic subunits (*a* and *a₃*). Complex IV (like mitochondrial complexes I and III) functions as an energy transducing site coupling to electron flow the the pumping of protons from the matrix to the intermembrane space thus fueling the ATP synthase (Wikstrom et al., 1981). Each subunit contains one heme center (heme *a* and heme *a₃*) associated with a copper center (*Cu_A* and *Cu_B*) which participate in a series of redox reactions which provide a pathway for electron flow from cytochrome c to molecular oxygen. In binding to heme *a₃*, oxygen undergoes reduction to two water molecules in a four electron process which consumes four protons (Michel, 1998). A proton is pumped across the membrane for each proton consumed in the terminal reaction. The reduction of oxygen catalysed by complex IV accounts for more than 90 % of all the O₂ consumed by living organisms. Cyanide, azide and CO strongly inhibit complex IV by binding tightly to the ferric form (Fe⁺⁺⁺) of heme *a₃*, which prevents it passing electrons to molecular oxygen.

1.2.10 Uncoupling Agents

Oxygen uptake, which is dependent on the presence of ADP and phosphate, is defined as coupled respiration. The coupling of respiration and phosphorylation can be dissociated by osmotic, physical (mechanical) or chemical disruption of the mitochondria by means of uncoupling agents.

Chemical uncouplers have the ability to shuttle H^+ across the inner mitochondrial membrane, resulting in dissipation of the H^+ gradient (Tyler, 1992). Uncouplers therefore isolate the processes of oxidation from phosphorylation in mitochondria. Since uncouplers collapse the H^+ gradient and prevent ATP synthesis, they overcome normal respiratory control, but cannot stop electron flow along the electron transport chain. Consequently respiration proceeds very rapidly because there is no chemiosmotic gradient to regulate electron flow.

Uncoupling agents are normally relatively small amphipathic molecules capable of high mobility in phospholipid bilayers and potent effects on proton permeability. Uncouplers shield the proton's electric charge as the ion passes through the membrane, creating a polar environment for the ion while achieving a hydrophobic interaction with lipids of the inner mitochondrial membrane (Tyler, 1992).

Carbonylcyanide phenylhydrazones (Heytler and Pritchard, 1962) are perhaps the most well-known uncouplers and this chemical group includes chlorocarbonylcyanide phenylhydrazone (CCCP) and the more potent trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP). They both act as H^+ ionophores as already discussed.

1.3 The Nerve and Neurotransmitter Release

1.3.1 The Neuron and its Function

A neuron represents a fundamental cell type in the nervous system. Neurons typically consist of four distinct domains, the dendrites, the cell body, the axon and the axon terminals or nerve endings. Each of these regions can be viewed as having separate functions. The cell body contains the nucleus together with many other organelles and the dendrites are fine projections (arborizations) of membrane and cytoplasm that are continuous with the soma and represent the main means that incoming electrical signals are picked up and relayed to the cell body. The axon is a single relatively long projection which relays action potentials from the cell body to the nerve ending. The axon hillock represents a region closest to the cell body which is the trigger zone for action potential propagation. The nerve ending then forms a synapse with the next neuron of the circuit.

Synapses are specialized structures where neurons send and receive information. A synaptic complex normally consists of three components, the presynaptic terminal, the synaptic cleft and the postsynaptic process. The axon terminal from the presynaptic cell sends signals that are picked up by postsynaptic cells. Two types of synapses are known, the electrical synapse (gap junction) and the chemical synapse, which have different morphology and function (Kandel and Siegelbaum, 1985; Bennett et al., 1991; Bennett and Scheller, 1993; Shepherd, 1994). Although electrical synapses link many sensory and motor neurons in invertebrate species, this type of synapse is relatively rare in mammalian nervous system. In electrical synapses, the gap junction connexin proteins form channels in the plasma membrane which allow ions to pass from the cytoplasm of one neuron into that of the adjacent neuron, achieving signal propagation (Kandel and

Siegelbaum, 1985). Chemical synapses on the other hand consist of pre- and post-synaptic membranes separated by a cleft of 30 - 50 nm, much wider than the approximately 2 nm gap between the junctional plasma membranes of electrical synapses (Kandel and Siegelbaum, 1985). Chemical synapses are the more abundant and elaborate form of junction in mammalian nervous system (Shepherd, 1994). The presynaptic and postsynaptic membranes of chemical synapses are often specialized and the presynaptic nerve endings contain numerous neurotransmitter-containing vesicles, which fuse with the pre-synaptic membrane to release neurotransmitters. The neurotransmitters then interact with specific postsynaptic specific receptors, which are coupled to ion channels generating various postsynaptic potentials (Kandel and Siegelbaum, 1985; Shepherd, 1994).

Synapses are classified as excitatory and inhibitory on the basis of the neurotransmitters contained within the pre-synaptic nerve ending forming the synapse. Excitatory synapses have nerve endings containing excitatory neurotransmitters such as glutamate, aspartate and acetylcholine. Action potentials invading the nerve ending open voltage-gated sodium channels increasing cytoplasmic Na^+ concentration. The depolarization caused by sodium ion influx triggers the opening of voltage-gated calcium channels and influx of Ca^{++} which activates presynaptic release of excitatory transmitter. Excitatory neurotransmitters diffuse rapidly to the postsynaptic membrane where they activate receptors coupled, for example, to sodium channels which depolarize dendritic regions of the post synaptic cell. Inhibitory synapses operate in a similar way but involve inhibitory chemical transmitters, such as γ -aminobutyric acid (GABA) which activates

postsynaptic GABA_A receptors causing influx of Cl⁻, which hyperpolarizes dendritic regions of the postsynaptic cell (Shepherd, 1994).

1.3.2. Neurotransmitter Substances

The three major categories of neurotransmitter include amino acids, amines and peptides. Glutamate, aspartate, glycine and GABA represent the most important amino acid-derived neurotransmitters. Acetylcholine (Ach), dopamine, epinephrine, norepinephrine (NE), histamine and serotonin (5-HT) are members of the amine group of neurotransmitters and prominent peptide neurotransmitters include substance P, neuropeptide Y, cholecystokinin, vasopressin, somatostatin, neuroensin, dynorphins and the enkephalins. The main excitatory and inhibitory neurotransmitters of the brain are glutamic acid and GABA, respectively. Glutamate is the most abundant excitatory amino acid neurotransmitter and widely distributed in mammalian brain, including the hippocampus (Kennedy, 1994; Conti and Weinberg, 1999). Aspartate is also excitatory but primarily confined to the ventral spinal cord and hippocampus (Fleck et al. 1993). After activating postsynaptic amino acid receptors, glutamate and aspartate are inactivated by carrier-mediated reuptake into the pre-synaptic terminal.

GABA is the major inhibitory neurotransmitter in the central nerve system of vertebrate brain accounting for transmission at 30 – 40 % of all synapses (Curtis and Johnston, 1974; Sivilotti and Nistri, 1991). The GABA concentration in the brain is 200 - 1000 times greater than that of the monoamines or acetylcholine, emphasizing the importance of GABA-ergic transmission in brain. GABA is synthesized from glutamic acid and its breakdown products are fed into the TCA cycle. Following release into the

synapse, GABA is inactivated by active transport into the presynaptic terminal and astrocytes (glial cells) that are closely associated with synapses.

Taurine is a sulfur-containing inhibitory amino acid neurotransmitter in brain (Huxtable, 1992). Taurine has been localized in the pineal gland and various centres involved in taste, smell and memory. This amino acid can affect acetylcholine levels, helping to reduce excitability in the nervous system during epilepsy. Taurine has also been shown to mimic GABA in activating GABA_A receptors, (Whitton et al., 1994), thus providing a another mechanism which might explain its inhibitory actions in the nervous system.

1.3.3 Involvement of Ca⁺⁺, Synapsins, and Ca⁺⁺/calmodulin-dependent Protein Kinases in Neurotransmitter Release

As explained previously, when an action potential depolarizes the nerve ending, voltage-gated calcium channels open causing Ca⁺⁺ influx. The increase in Ca⁺⁺ triggers synapsin phosphorylation (Kelly, 1988). Synapsins are proteins capable of interacting both with the cytoplasmic surface of synaptic vesicles and protein kinases. When the cytosolic levels of Ca⁺⁺ increase, Ca⁺⁺ binds to calmodulin (a small soluble protein) forming the Ca⁺⁺/cam complex, which then activates calcium/calmodulin-dependent protein kinase II (Ca⁺⁺/cam kinase II). The activated Ca⁺⁺/cam kinase II phosphorylates synapsins which cause release of synaptic vesicles from the cytoskeleton and facilitate vesicle docking with the presynaptic membrane and release of neurotransmitters (Greengard et al., 1993; Zimmermann, 1993). After release of neurotransmitters,

phosphatases then dephosphorylate synapsin molecules, which allowing them to bind to the surface of synaptic vesicles, actin and fodrin and resecure vesicles to the cytoskeletal framework within the pre-synaptic terminal (Trimble et al., 1991). The phosphorylation and dephosphorylation cycle within the nerve ending is therefore intimately involved in regulation of transmitter release. The SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) hypothesis can explain the docking, fusion and release of vesicles in synaptosomes (Brunger, 2001a; Fiebig et al., 1999). Two proteins, NEM-sensitive fusion protein (NSF) and soluble NSF-attachment proteins (SNAPs) (Malhotra et al., 1988; Clary et al., 1990), are involved in synaptic vesicle fusion. These proteins interact with vesicle-associated SNARE (v-SNARE), such as synaptobrevin, and target membrane SNAREs (t-SNARE), syntaxin and SNAP-25, to form a macromolecular complex that spans the two membranes and causes them to fuse (Lin and Scheller, 2000). Synaptotagmin, another vesicle-associated protein, acts as a Ca^{++} sensor, and causes vesicle release when Ca^{++} binds (Brunger, 2001b; Sudhof, 2004).

1.4 Absorbance and Fluorescence Methods

Since many of the assays I used in my thesis research involved either absorbance or fluorescence determinations, this section briefly describes the principles behind these techniques.

UV-visible spectroscopy is the measurement of the wavelength and intensity of absorption of ultraviolet and visible light by a sample. When an atom or molecule absorbs light energy, electrons are promoted from their ground state to an excited state. A large number of molecules absorb ultraviolet or visible light, however, the absorption in

organic molecules is restricted to certain functional groups called chromophores. Different chromophores absorb radiation of different wavelengths often due to the presence of an aromatic or conjugated system in the molecule. Since absorption involves measurement of transmitted light relative to incident light intensities at the same wavelength, the concentration of an analyte in solution can be determined. The solvent or some compounds also have an effect on the spectrum of the species. The red shift in absorbance of complex III is caused by attractive polarization forces between the solvent and the absorber, which lower the energy levels of both the excited and unexcited states. This effect is greater for the excited state, and so the energy difference between the excited and unexcited states is slightly reduced, resulting in a small red shift.

Fluorescence occurs in certain molecules (fluorophores) when energy, absorbed from an external light source, excites electrons from a vibrational level in the electronic ground state to one of the many vibrational levels in the electronic excited state. The excited electronic singlet state then rapidly and spontaneously falls to the lower ground state and, as a result of this process, energy is dissipated in the form of a photon of light. Since the energy of this photon is lower than that of the excitation photon, its wavelength is longer. Fluorescence quantitation of OPA-amino acids was used extensively to investigate the transmitter-releasing effects of surangin B.

1.5 Objectives of this Research

Plants contain a large reservoir of pesticidal substances that may be used directly or as prototypes for the synthesis of pesticides. The interest of the pesticide industry and the pesticide market in this source of natural products as pesticides has increased

dramatically in the past few years. The published research on the botanical coumarin surangin B (discussed in chapter 1.1) would suggest that the pesticidal properties of this and related coumarins warrant systematic investigation. Recent regulatory requirements increasingly require adequate definition of mechanism of action as well as definition of toxic effects and this applies to natural products as well as synthetic pesticides.

The research described in this thesis therefore seeks to clarify firstly the nature of surangin B's interaction with mitochondria, and secondly the ability of surangin B to cause endogenous release of a key excitatory (L-glutamate) and inhibitory (GABA) neurotransmitter in brain. Since these results showed that surangin B acted in a similar way to some commercial fungicides, my third objective was to investigate the potential antifungal action of surangin B.

For the first and second objectives, my experiments used preparations from bovine heart mitochondria and mouse brain synaptosomes (functional pinched-off nerve endings), respectively, and particular attention was given to delineating the mechanism of action of surangin B in this research. In antifungal experiments, the effects of surangin B towards several fungal pathogens of crop plants were compared with other antifungals including commercial fungicides. The detailed approaches taken to address each research objective are described in Chapters 2 - 4 that follow.

CHAPTER 2. INTERACTION OF SURANGIN B WITH BOVINE HEART MITOCHONDRIA

2.1 Introduction

As already discussed in Chapter 1 under "Insecticidal Coumarins" certain of the coumarins in *Mammea americana* and especially surangin B, a 4-(1-acetoxypyrpyl)-containing coumarin of *Mammea longifolia*, show interesting insecticidal activity. Surangin B does not exhibit early knockdown in insects, but instead causes an ever deepening paralysis, which is similar to the symptomology of some compounds which target mitochondria (Zheng et al., 1998). Zheng and colleagues (1998) found that surangin B is a very potent blocker of state 3 respiration when insect muscle mitochondria are respiring on NAD^+ - or FAD^+ - linked substrates, but this coumarin has minimal effect on mitochondrial complexes I and IV. These results show that in insects, complexes II or III may be relevant targets of this coumarin. Other previous work from our laboratory has found that surangin B inhibits oxygen uptake by mouse brain synaptosomes and depolarizes intrasynaptosomal mitochondria, an effect also produced by the specific complex I inhibitor rotenone (Nicholson and Zhang, 1995). It is likely that mitochondrial blockade leading to bioenergetic failure in muscle and nerve, the latter causing indiscriminate release of neurotransmitters, is responsible for the paralysis in insects exposed to surangin B.

In an attempt to resolve questions on the sensitivity of the various mitochondrial complexes to surangin B and to examine some of the mechanisms involved, I initiated a

systematic investigation into the action of surangin B in the model beef heart mitochondrial preparation. My first objective in this study was to investigate the sensitivity of the different complexes of the electron transport chain of mammalian mitochondria to surangin B to determine which may be its relevant target(s). Knowing this, I would embark on an investigation to clarify the mechanism(s) involved.

2.2 Materials and Methods

2.2.1 Chemicals and Biological Materials

Surangin B was extracted and purified from the roots of *Mammea longifolia* according to a method described by Joshi et al. (1969). Nicotinamide adenine dinucleotide (reduced form; NADH), sodium succinate, 2,6-dichlorophenolindophenol (DCIP), cytochrome C, rotenone, antimycin A (AA), myxothiazol, thenoyltrifluoroacetone (TTFA), nitropropionic acid (NP), sodium cyanide, sodium borohydride and decylubiquinone were purchased from Sigma-Aldrich Canada Ltd. Oakville (ON). Carboxin and famoxadone were kindly provided by Dr. Mark Dekeyser, Uniroyal Chemical Ltd, Guelph, (ON) and Dr. Douglas Jordan, Sine Haskell Research Center, Newark, (DE), respectively. Bovine hearts were obtained from Grand Maison Beef Farm Ltd., Cloverdale (BC). All other solvents and reagents employed in this study were analytical grade.

2.2.2 Isolation of Mitochondria from Bovine Heart

A bovine heart, obtained within five minutes of animal slaughter, was sealed in a plastic bag and immediately transferred to large cooler containing crushed ice for transport to the lab. Upon arrival at the laboratory (within 1 hour), adipose and connective tissue were removed as much as possible and the heart muscle was then sectioned into small cubes (approximately 1 x 1 cm). Heart tissue was placed in ice-cold 0.25 M sucrose solution, containing 0.01 M tris base and 0.2 mM ethylenediaminetetraacetic acid (EDTA), adjusted to pH 7.8 with HCl, and blended (1 + 2; w/v) for 35 - 40 sec. After adjustment of the pH to 7.8 with tris base (2 M), the mixture was homogenized in batches at approximately 1,200 rpm pestle rotation using two 10 second passes. This homogenate was again adjusted to pH 7.8 using 2 M tris base prior to centrifugation (Beckman J2-HS) at 1,200g for 20 min. The supernatant was carefully removed and filtered through two layers of cheesecloth. The filtrate was then adjusted to pH 7.8 with 2 M tris base and centrifuged (Beckman J2-HS) for 15 min at 26,000 g. The upper portion of the pellet (loosely compacted buff-colored layer), consisting of broken mitochondria, was discarded. The middle dark brown layer (consisting of intact mitochondria) was resuspended in sucrose solution (10 ml; using homogenization), added to a further 170 ml of ice-cold sucrose solution and the pH re-adjusted to 7.8. The suspension was centrifuged at 26,000g for 15 min. The mitochondrial pellet was washed again by resuspension and centrifugation yielding the final pellet which was suspended in sucrose solution to a protein concentration of 20 - 40 mg/ml, then aliquoted into vials and flash frozen in liquid nitrogen. Mitochondria were stored at - 80 °C prior to experimentation. All experiment and centrifugation procedures were carried out at 1 - 5 °C.

2.2.3 Preparation of Submitochondrial Particles (SMPs)

Submitochondrial particles were prepared from bovine heart mitochondria using the procedure of Matsuno-Yagi and Hatefi (1985). Frozen mitochondria were allowed to thaw slowly at room temperature. Batches of the suspension (100 ml) were then homogenized and subjected to sonication for 1 min at 0 °C using Branson sonicator. The sonication was repeated again after allowing the samples to cool down for 5 min in an ice bath. After this, the pH was adjusted to 7.5 with 1 M KOH and the suspension was centrifuged for 7 min at 32,500 g. The supernatant was carefully removed leaving behind a loosely packed pellet which was then compacted by recentrifugation for 45 min at 130,000 g. The pellet was washed once by resuspension in 10 mM tris-acetate buffer pH 7.5, containing 0.25 M sucrose followed by centrifugation. The final submitochondrial particle pellet was suspended in the same buffer at a protein concentration of 40 - 60 mg protein/ml, frozen in liquid nitrogen in small aliquots, and stored at -80 °C.

2.2.4 Assays of Complexes I to IV

Each complex (I - IV) was assayed spectrophotometrically (Spectronic 3000 Array Spectrophotometer, Milton Roy Company) using previously published techniques (Krahenbuhl et al., 1991; Desai et al., 1996) with minor modifications. Complex I activity was measured by following the oxidation of NADH, given by a decrease in absorbance at 340 nm. The reaction mixture consisted of 50 mM potassium phosphate buffer (1 ml; pH 7.6) containing 0.25 mM NADH and decylubiquinone (50 µM) as the electron acceptor. Mitochondria (200 µg protein) were added to start the reaction. Complex II activity was measured as the rate of reduction of ubiquinone to ubiquinol by succinate, and

quantitated by the secondary reduction of 2,6-dichlorophenolindophenol (DCIP) as the quinol forms. The reaction mixture contained 50 mM potassium phosphate buffer (1 ml; pH 7.6), 20 mM succinate, 1.0 mM EDTA, 0.05 mM DCIP and 3 mM sodium azide. Decylubiquinone (50 μ M) was added followed by mitochondria (65 μ g) to start the reaction. The decrease in absorbance as DCIP becomes reduced was measured at 600 nm. Complex III activity was assayed as an increase in absorbance at 550 nm as cytochrome *c* is reduced by complex III with decylubiquinol present as the electron donor. The reaction mixture consisted of 50 mM potassium phosphate buffer (1 ml; pH 7.6) containing 0.1 % BSA, 0.1 mM EDTA, 60 μ M cytochrome *c*, 3 mM sodium azide, and decylubiquinol (150 μ M). Mitochondria (10 μ g protein) were then added to initiate the reaction. Complex IV activity was measured with reduced cytochrome *c* as substrate. The reaction mixture consisted of 50 mM potassium phosphate buffer (1 ml; pH 7.6) containing sucrose (0.25 mM) and 25 μ M reduced cytochrome *c*. Mitochondria (10 μ g protein) were introduced to start the reaction and the oxidation of cytochrome *c* was monitored at 550 nm. Cytochrome *c* was reduced with sodium dithionite prior to assay. Inhibitors were added to these assays using microsyringes in no more than 3 μ l DMSO and all assays were carried out at 30 °C.

2.2.5 Effect of Surangin B on Complex II Kinetics

Complex II oxidation was measured at 30°C using succinate as a substrate and DCIP as a terminal electron acceptor (Mowery, et al., 1976; White, 1971). The reaction mixture (total volume 1 ml) contained 1.0 mM EDTA; 0.05 mM DCIP and 3 mM NaN₃ in 50 mM potassium phosphate buffer (pH 7.6). In the first series of experiments, the

succinate substrate was added at different concentrations (5, 10, 15, 20 and 25 mM) and decylubiquinone was kept constant at (50 μ M). In the second set of assays the complex II electron acceptor analog decylubiquinone was varied (5, 7.5, 15 and 25 μ M) and succinate was held at 20 mM throughout. Surangin B and carboxin were introduced in DMSO (10 μ l) and malonate was added in buffer. Submitochondrial particles (45 μ g) were added to initiate the reaction and the absorbance decline was followed over 5 minutes at 600 nm.

2.2.6 Purification and Characterization of Complex III

The isolation of complex III from beef heart mitochondria used the method of Berry et al., (1991) with slight modifications. Upon thawing, mitochondria were added to ice-cold 50 mM potassium phosphate buffer (pH 7.5) containing 300 mM NaCl and 1 % dodecyl maltoside to achieve a final concentrations of 1 g/100 ml protein. Gentle homogenization was used to assist solubilization of mitochondrial material and the resulting suspension was centrifuged at 130,000g in a Beckman L8-80 ultracentrifuge for 25 min. The supernatant was applied to a DEAE-Sepharose column (1.5 x 50 cm) and moved into the upper stationary phase with 50 mM potassium phosphate buffer, pH 7.5, containing 300 mM NaCl and 0.01 % dodecyl maltoside. The detergent-solubilized mitochondrial extract was then eluted (15 ml/hr) with 50 mM potassium phosphate buffer (pH 7.5) and 0.01 % dodecyl maltoside incorporating a linear gradient (150 + 150 ml) of 300 - 500 mM NaCl. Fractions (5 ml) were collected and assayed by protein determination (Figure 2.1). Complex III (contained in red fractions) was then adsorbed onto a 1 x 2 cm hydroxyapatite column and washed with 50 mM potassium phosphate

buffer (pH 7.5) containing 300 mM NaCl and 0.01 % dodecyl maltoside (Berry et al., 1991). Purified complex III was then eluted using 300 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA, and 0.1 % Brij-35 detergent, allowing isolation of the product in 1 - 2 ml. Aliquots of this concentrated solution of complex III were taken for protein determination, assay of enzymic activity (Fig 2.2) and PAGE analysis (Schagger and von Jagow, 1987) (15 % gel was used in this experiment) to confirm identity and purity (Fig 2.3). The remainder was flash frozen in liquid nitrogen and stored at -80°C until used.

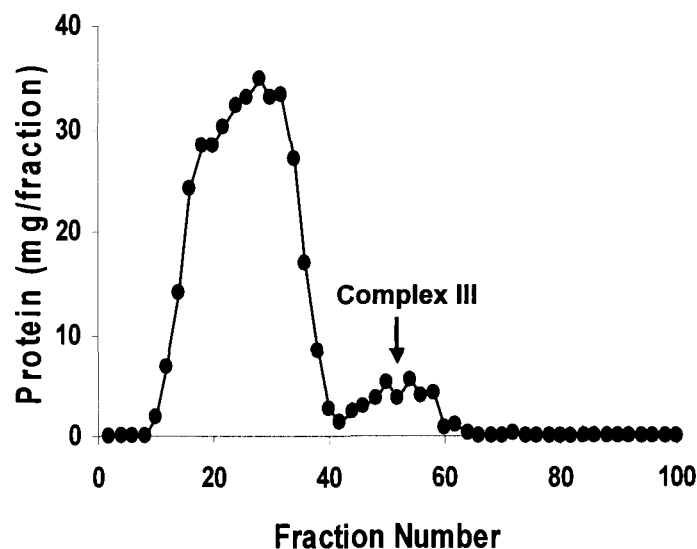


Figure 2.1 Chromatography of dodecylmaltoside-solubilized bovine Heart mitochondria on DEAE-Sephacrose.

2.2.7 Assay of Red Shift Difference Absorption Spectra

Recordings of red shift spectra were conducted on purified complex III using a Milton Roy Spectronic 3000 Array as described by Jordan et al., (1999). Inhibitors were added singly or sequentially (as required) to complex III (0.215 mg protein/ml) following its complete reduction with sodium dithionite. Complex III was scanned (500 - 650 nm) after each addition and the result was recorded digitally. The difference spectra were calculated by subtraction of digitally-stored spectra from the various complex III treatments as appropriate. Inhibitors were added in DMSO (3 μ L).

2.2.8 Synthesis of Decylubiquinol

Decylubiquinol was synthesized according to of Gudz (Gudz et al., 1997) and all procedures were carried out in subdued light. Decylubiquinone (10 μ mol) was dissolved in 2 ml ethanol+water (1 + 1 v/v; pH 2) and reduced to the corresponding alcohol by addition of NaBH₄. The decylubiquinol was twice extracted from the aqueous ethanol using 1 ml of diethylether+isooctane (2 + 1; v/v). The organic phases were combined, then washed with 2 ml of 2 M NaCl and evaporated to dryness at room temperature under a stream of nitrogen. The product was dissolved in ethanol (990 μ l), acidified by addition of 10 μ l of 0.1 M HCl and transferred to a storage vial and kept at - 20 °C in darkness.

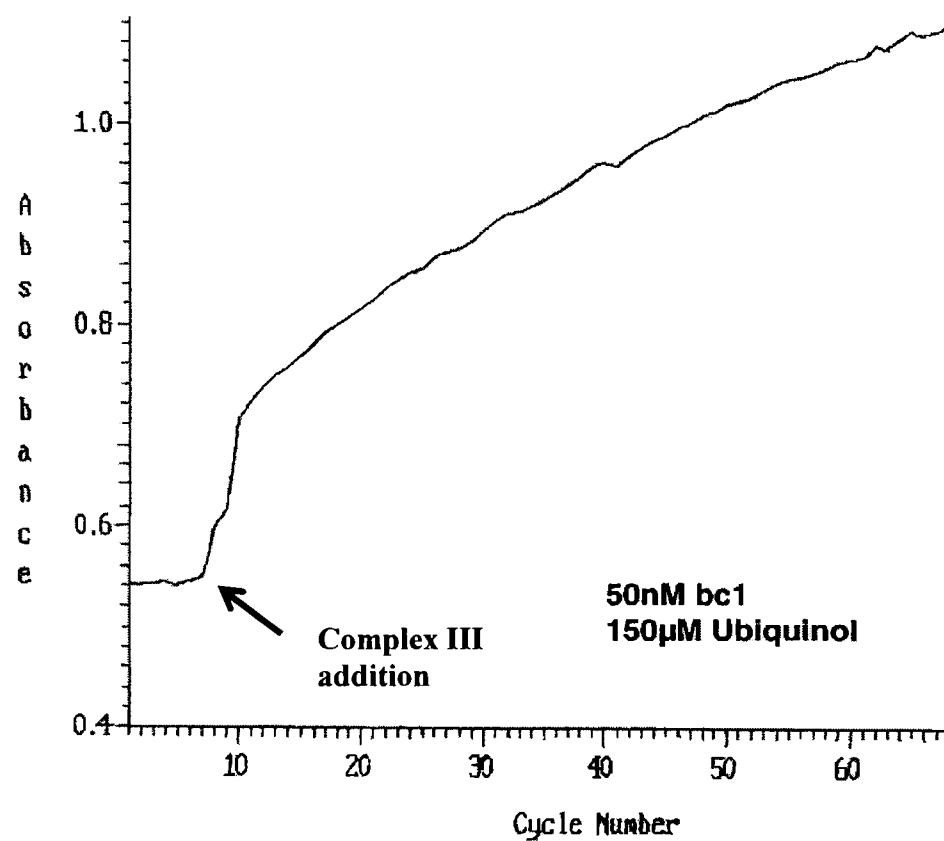


Figure 2.2 Cytochrome reductase activity of purified complex III. Absorbance measurement were carried out at 550 nm. Each cycle is 3 seconds.

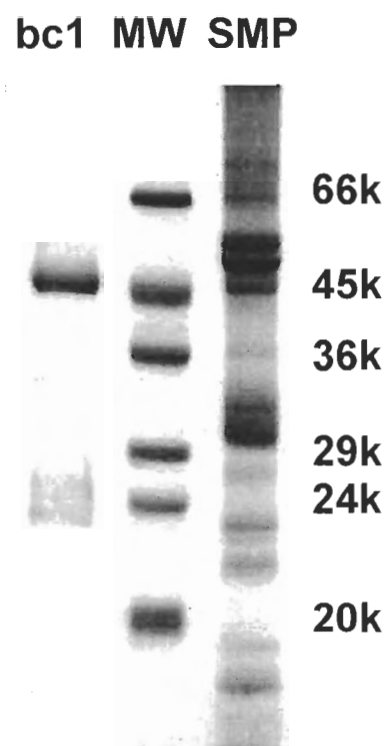


Figure 2.3 SDS-PAGE analysis of complex III isolated from bovine heart. (bc1 = complex III; SMP = submitochondrial particles; MW = molecular weight)

2.2.9 Synthesis of 2-nitrosofluorene (2-NOF)

The procedure I employed for synthesis of 2-NOF (Fig. 2.4) was based on an approach developed by Lotlikar et al., (1965) and Sandler et al. (1971) with modification. 2-Nitrofluorene (1 g) was dissolved in 100 ml dimethylformamide (DMF). To this was added 0.4 g ammonium chloride dissolved in 100 ml of 80 % ethanol, followed by 1g zinc dust and the mixture stirred overnight at room temperature. The reaction vessel was continuously flushed with nitrogen gas. After filtration (Whatman No. 1), the solid material was washed with 200 ml DMF + distilled water (4 + 1, v/v). Next, 25 ml of concentrated H_2SO_4 was slowly added to the filtrate, followed by sodium dichromate (0.45 g) in 1.5 ml distilled water. The combination was then stirred for 1 - 2 hrs over an ice bath after which ice-cold distilled water (approximately 1 litre) was slowly added. The precipitate of 2-nitrosofluorene was harvested by centrifugation at 2,000 g for 5 min and dried under vacuum.

UV absorbance measurements on 2-nitrosofluorene in 95 % ethanol gave the expected absorption spectrum maxima at 362 and 246 nm; minima at 280 and 227 nm and the shoulder at 260 nm (Fig 2.5). The crystal melting point was found to be 78 - 79 °C, in agreement with Lotlikar et al., (1965). Purity was found to be at least 98 % (Fig 2.6 a and b) using reverse phase (C_{18}) HPLC with two different mobile phases: (1) acetonitrile + 1 % phosphoric acid (70 + 30 v/v); (2) a linear gradient of acetonitrile 0 – 100 % over 30 min. in 0.1 % acetic acid with absorbance detection at 360 nm at room temperature.

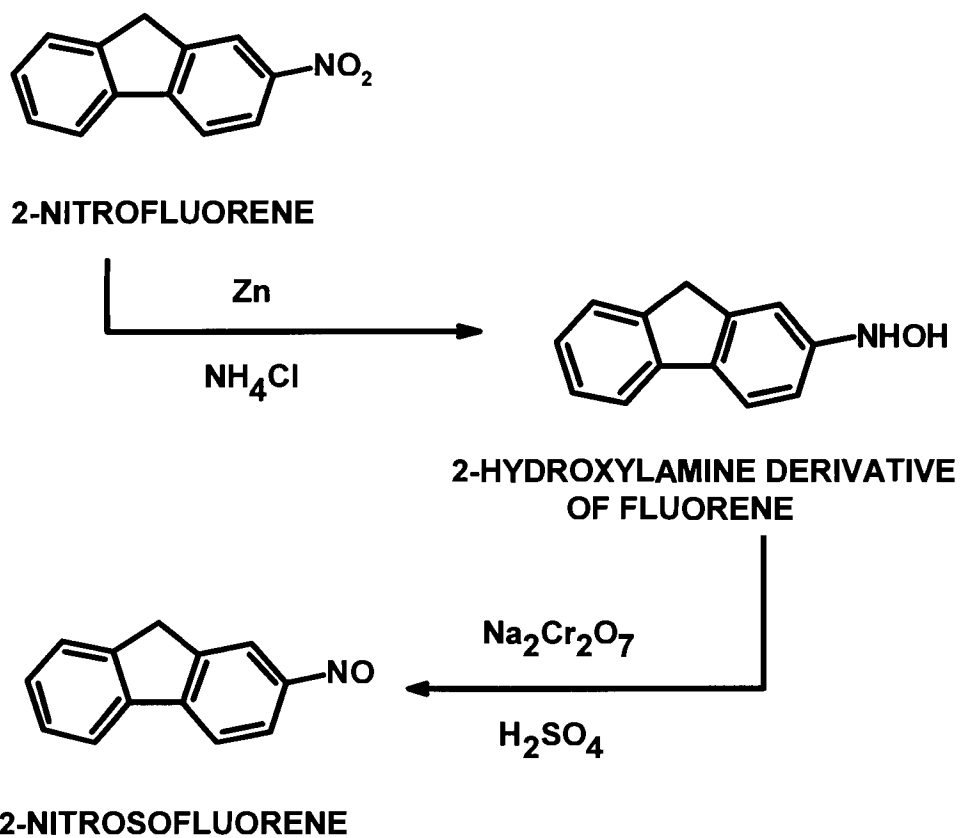


Figure 2.4 Synthesis of 2-nitrosofluorene.

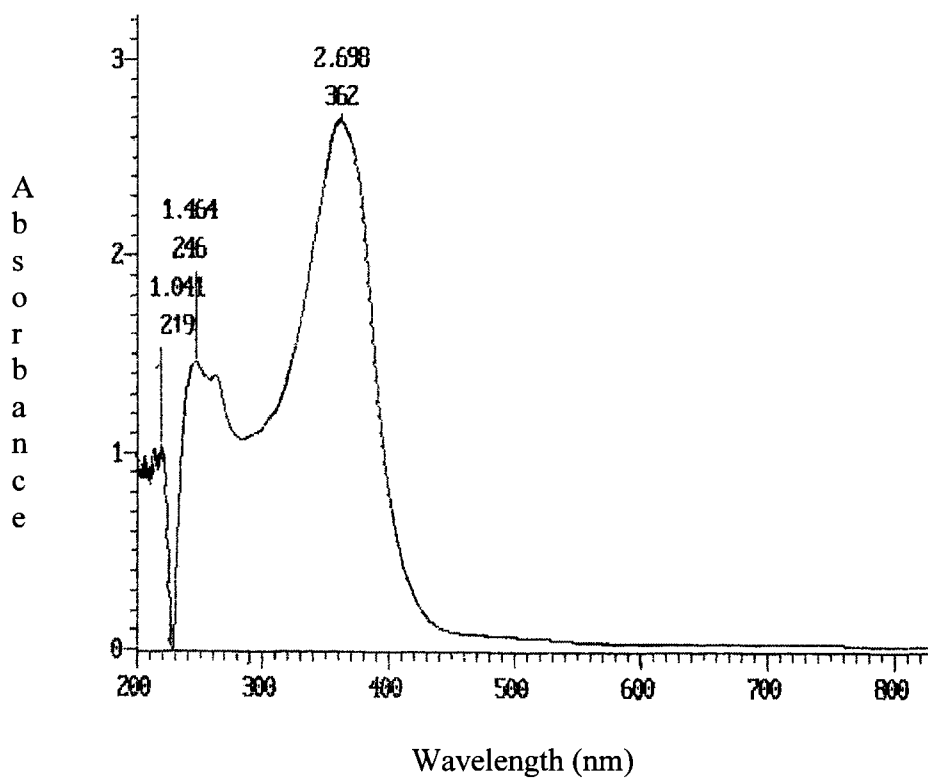


Figure 2.5 The UV spectrum of 2-NOF. The expected absorption spectrum maxima at 362 and 246 nm; minima at 280 and 227 nm and the shoulder at 260 nm are present.

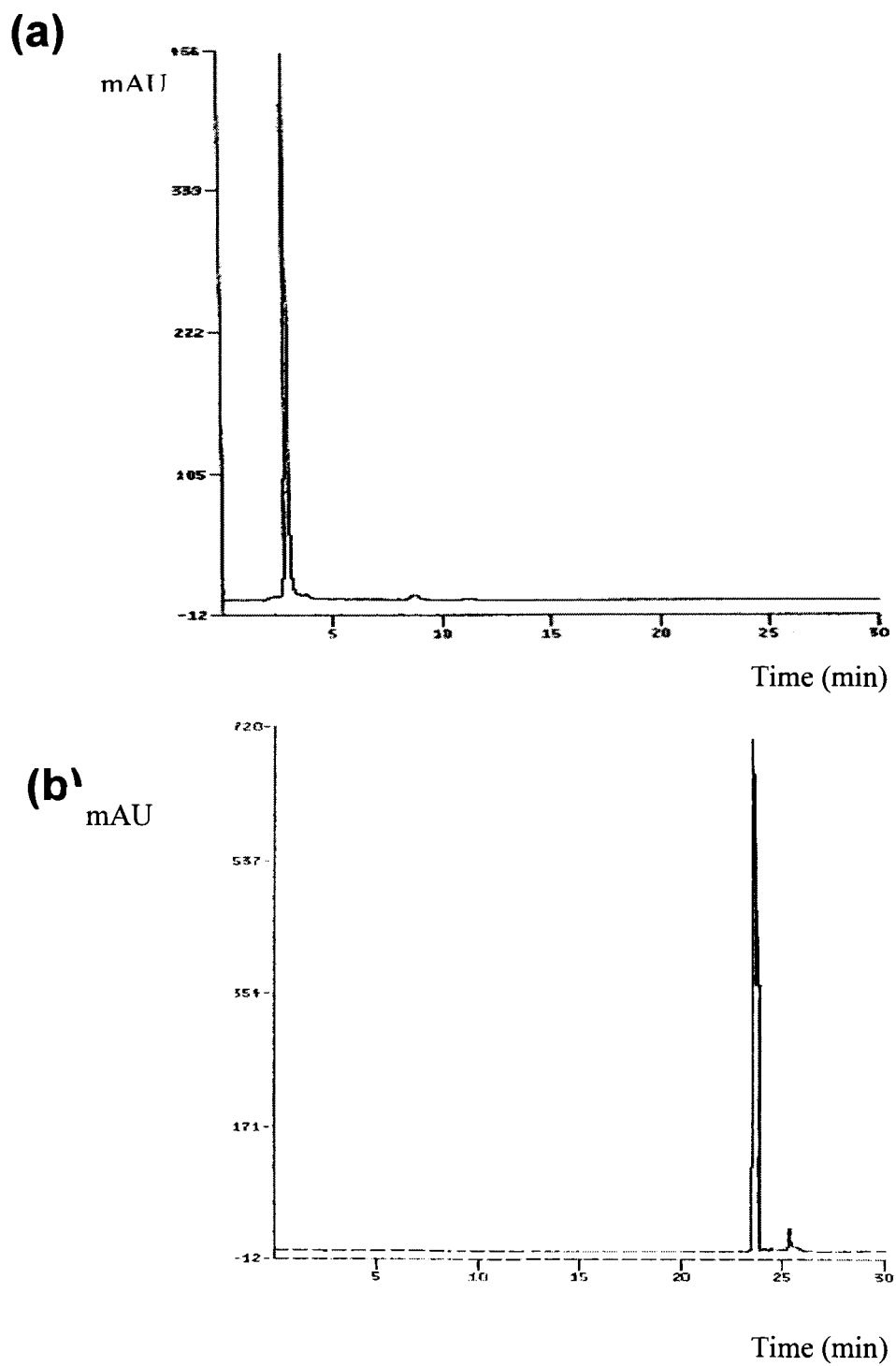


Figure 2.6 RP-HPLC traces of synthetic 2-NOF. (a) acetonitrile + 1 % phosphoric acid (70 + 30 v/v); (b) a linear gradient of acetonitrile 0 – 100 % over 30 min. in 0.1 % acetic acid with absorbance detection at 360 nm at room temperature.

2.2.10 Assay of 2-NOF-stimulated Oxygen Consumption by Mitochondria

Oxygen consumption of beef heart mitochondria was measured at 25 °C in a 2 ml glass chamber using a Clark-type oxygen electrode (Yellow Springs Instrument Co. Inc., OH) fitted with a high sensitivity membrane using a technique described by Klohn et al. (1996). Mitochondria (1.8 mg) were stirred continuously in respiration buffer (0.3 M sucrose, 5 mM Na-MOPS, 1mM EGTA, 5 mM KH₂PO₄, 5 mM MgSO₄·7H₂O and 1 % BSA (fatty acid free), adjusted to pH 7.4 with NaOH. The output from the electrode was amplified and oxygen consumption recorded using LABVIEW 4.0 (National Instruments Corporation, Austin, TX). Surangin B, other inhibitors and 2-nitrosofluorene, were added in no more than 3 µl DMSO.

2.2.11 Protein Assays

Protein concentrations in whole mitochondrial preparations were conveniently determined using the biuret method with bovine serum albumin as a standard (Gornall et al., 1949). All other protein estimations were carried out using an adaptation of the Lowry procedure (Peterson, 1977).

2.2.12 Statistical Analyses

Data were analyzed by analysis of variance (ANOVA), followed by Student's t-test. A value of $p < 0.05$ was taken as significant and $p < 0.01$ as highly significant. The

concentration causing 50% inhibition of the response (IC_{50}) was calculated using Prism 3 software (GraphPad Software Inc., San Diego, CA).

2.3 Results

2.3.1 Differential Effects of Surangin B at Complexes I - IV of Bovine Heart

Mitochondria

In bovine heart mitochondrial preparations, concentrations of surangin B as high as 100 μ M are unable to inhibit complex I (Fig. 2.7 and 2.8), as compared to the classical complex I inhibitor rotenone, which produced threshold inhibition of complex I at 1 nM and 90 % inhibition at 1 μ M (Fig 2.8). However, in obvious contrast, surangin B is a potent inhibitor of complex II (IC_{50} = 0.2 μ M; Fig. 2.7). In parallel assays, the selective complex II inhibitors carboxin, oxy-carboxin, thenoyltrifluoroacetone and nitropropionic acid also showed extensive block whereas the negative controls rotenone, antimycin A, famoxadone and cyanide were ineffective (Fig. 2.9). Bovine heart mitochondrial complexes III and IV were also sensitive to inhibition by surangin B (IC_{50} s 14.8 μ M and 3.1 μ M respectively; Fig 2.7) under conditions where positive controls famoxadone and antimycin (complex III) and cyanide (complex IV) gave the expected inhibition (Fig. 2.10 and 2.11).

2.3.2 Inhibitory Kinetics of Surangin B on Complex II

Kinetic studies on surangin B's action at complex II of bovine heart mitochondria. Figure 2.12 (a, b and c) includes velocity versus succinate substrate plots as influenced by

a) surangin B, b) carboxin and c) the classical competitive inhibitor malonate. Surangin B inhibits succinate oxidation with mixed type kinetics. Consistent with the literature, malonate and carboxin are respectively competitive and non-competitive inhibitors of succinate oxidation by complex II (White, 1971). The double reciprocal plots for inhibition of complex II showed non-competitive inhibition with respect to decylubiquinone for surangin B (Fig. 2.13 a), carboxin (Fig. 2.13 b) and malonate (Fig. 2.13 c).

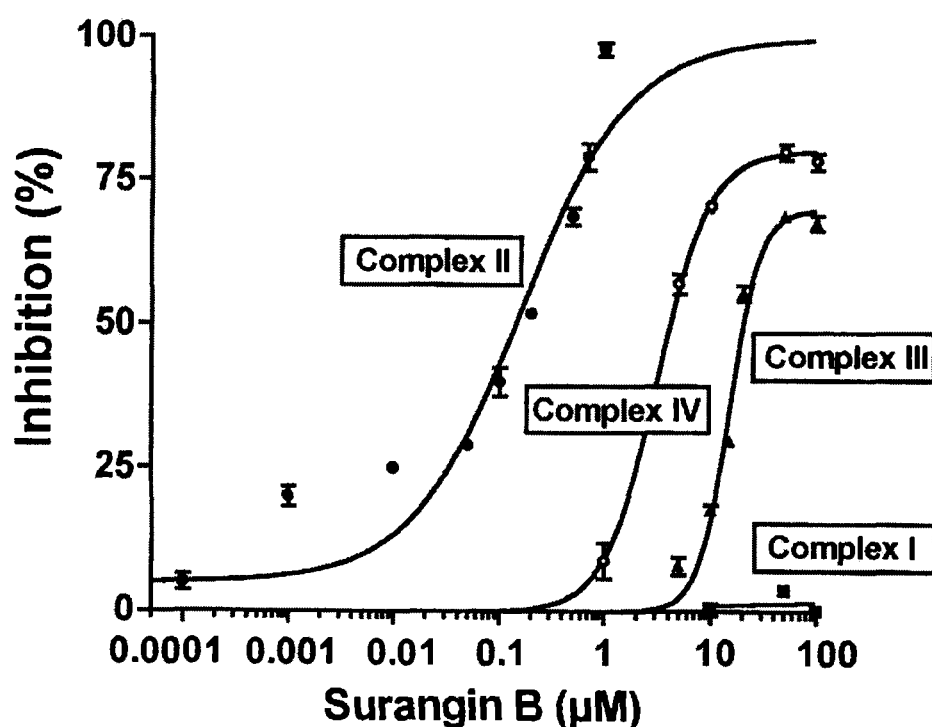


Figure 2.7 Concentration-dependence of surangin B's inhibitory effects on electron transport at complexes I, II, III and IV of bovine heart mitochondria. Data points show mean \pm standard error of 3 - 5 determinations.

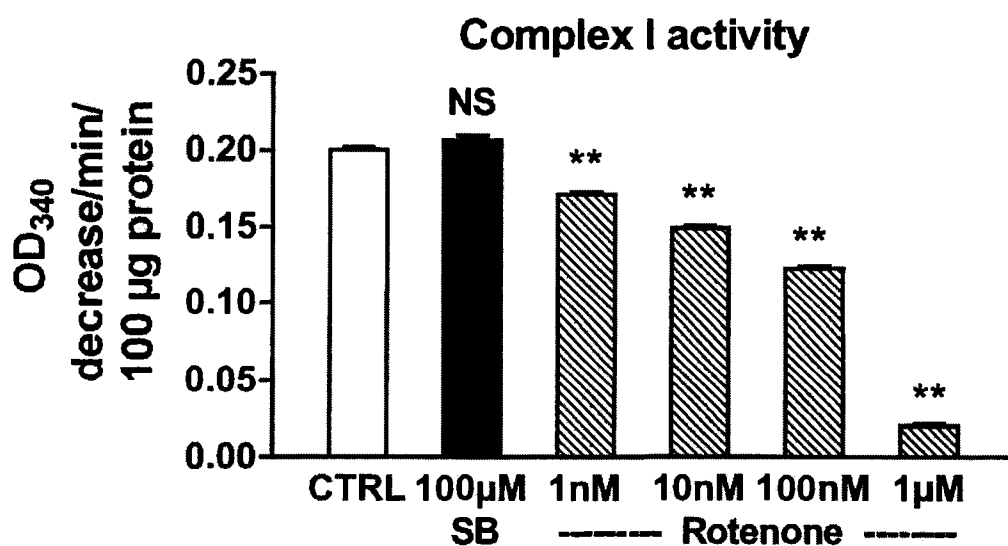


Figure 2.8 Comparison of surangin B with rotenone in assays of complex I using bovine heart mitochondria. SB = surangin B. Columns represent means and the bars the standard errors of at least 3 determinations.

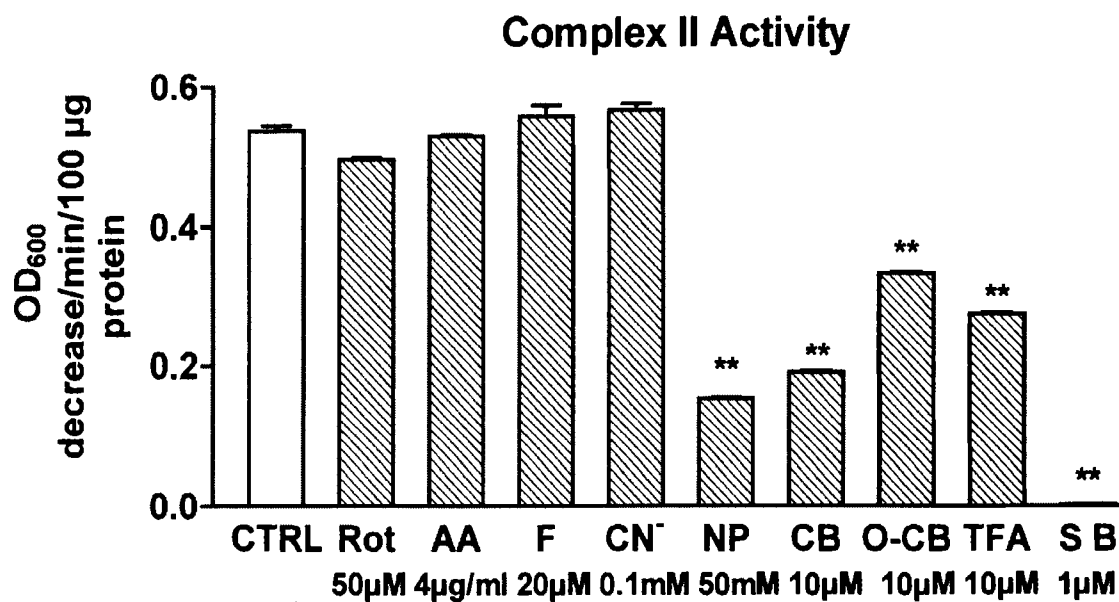


Figure 2.9 Comparison of surangin B with other inhibitors of electron transport in assays of complex II using bovine heart mitochondria. SB = surangin B, Rot = rotenone, F = famoxadone, CN⁻ = cyanide, NP = nitropropionic acid, TFA = thenoyltrifluoroacetone, CB = carboxin, O-CB = oxycarboxin, AA = antimycin A. Columns represent means and the bars the standard errors of at least 3 determinations.

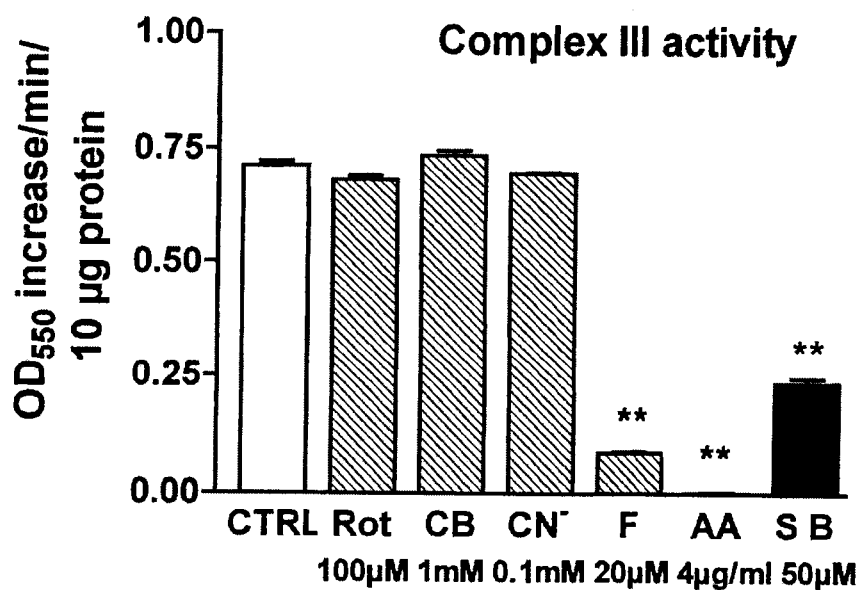


Figure 2.10 Comparison of surangin B with other inhibitors of electron transport in assays of complex III using bovine heart mitochondria. SB = surangin B, Rot = rotenone, F = famoxadone, CN⁻ = cyanide, CB = carboxin, AA = antimycin A. Columns represent means and the bars the standard errors of at least 3 determinations.

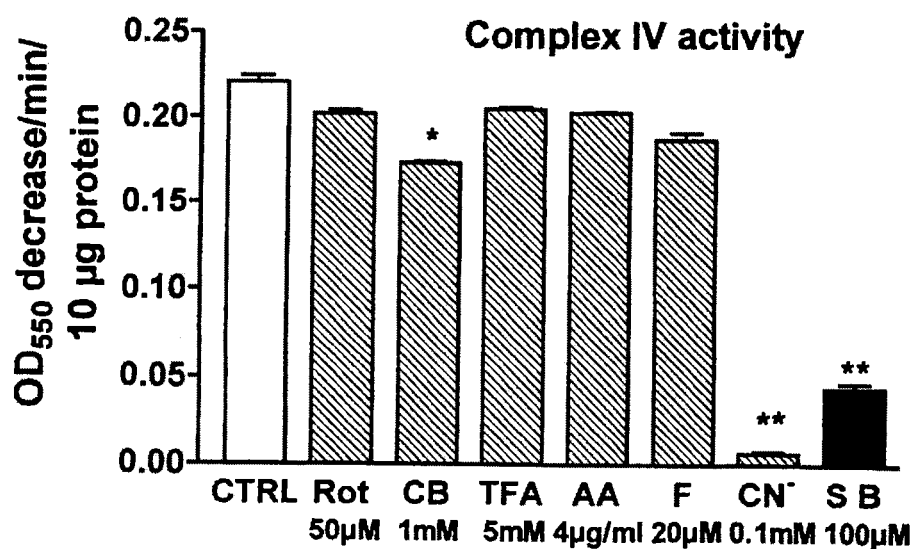


Figure 2.11 Comparison of surangin B with other inhibitors of electron transport in assays of complex IV using bovine heart mitochondria. SB = surangin B, Rot = rotenone, F = famoxadone, CN⁻ = cyanide, TFA = thenoyltrifluoroacetone, CB = carboxin, AA = antimycin A. Columns represent means and the bars the standard errors of at least 3 determinations.

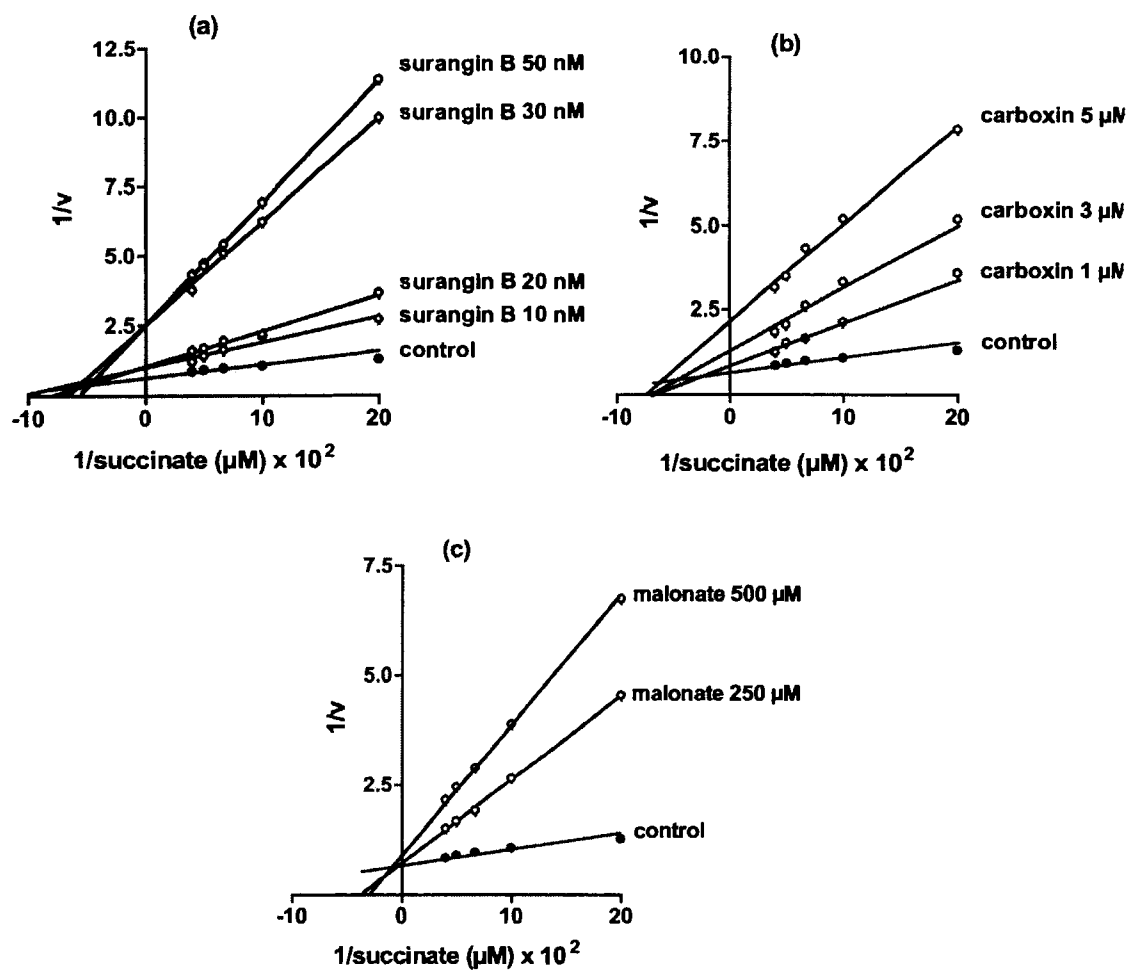


Figure 2.12 Double reciprocal plots showing the effect of increasing concentration of surangin B on complex II using different concentrations of the electron donor succinate compared to equivalent experiments with the inhibitors carboxin and malonate. Data points represent means and the bars the \pm standard errors of 3 - 6 determinations.

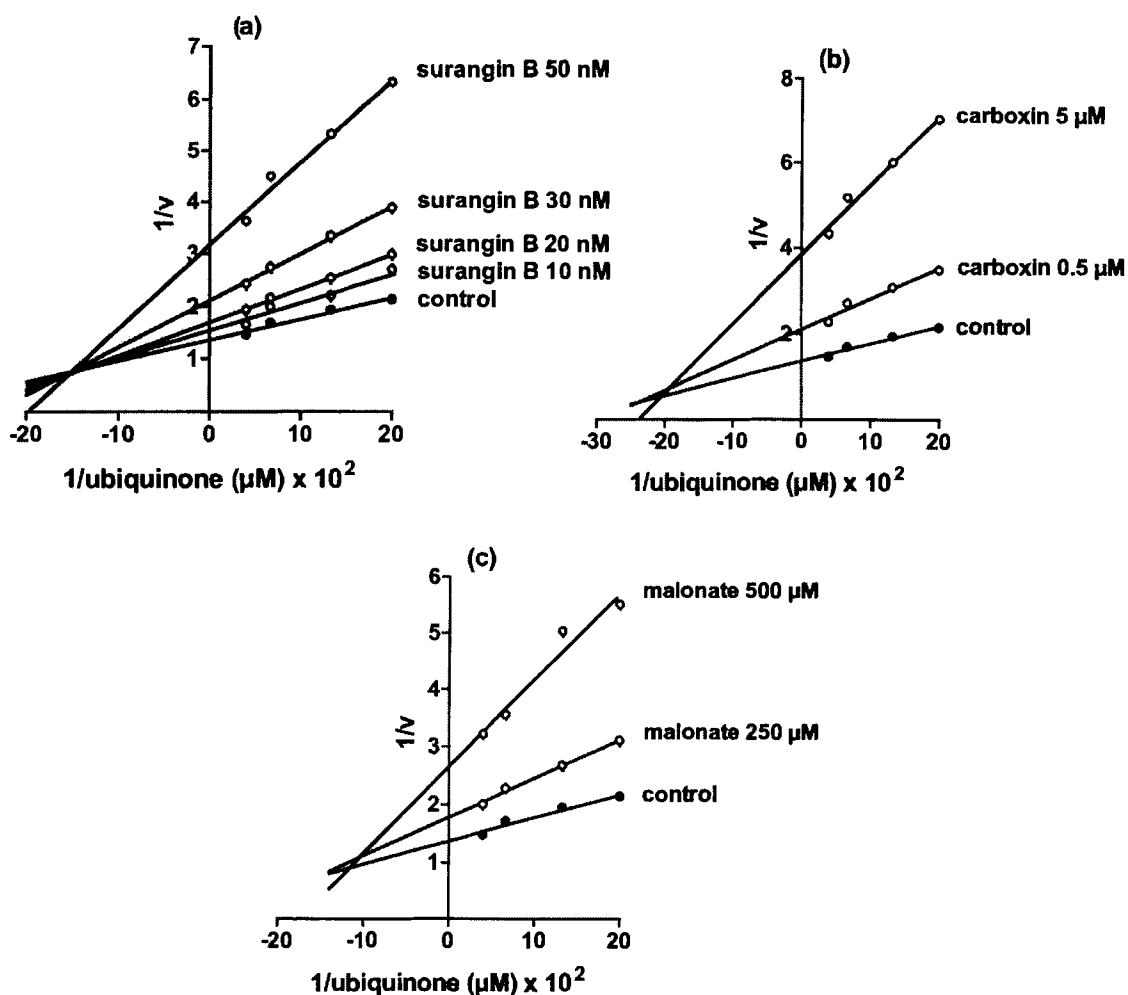
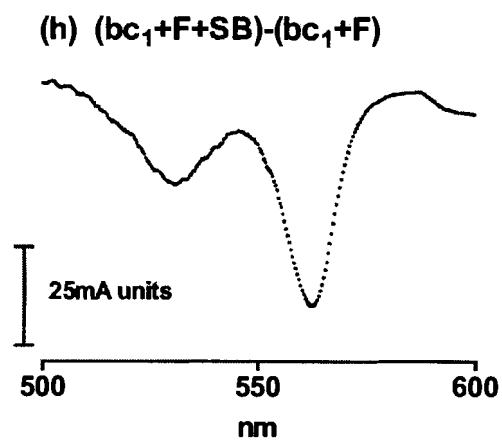
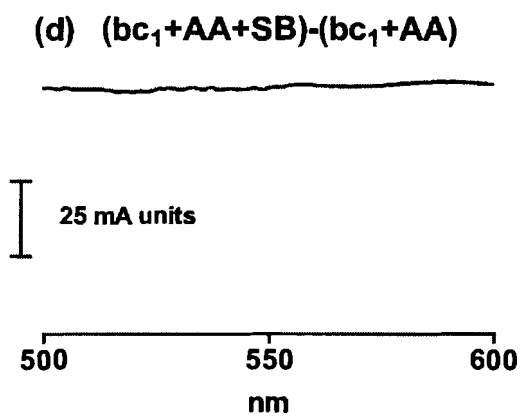
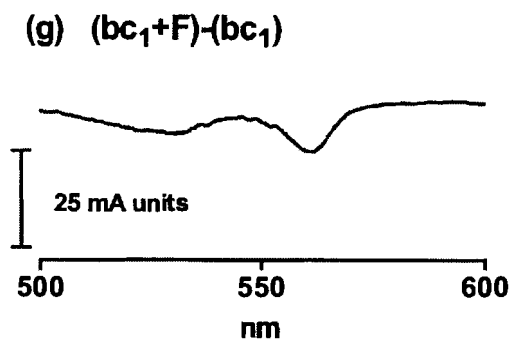
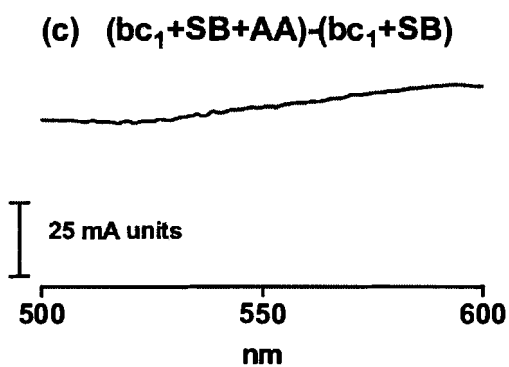
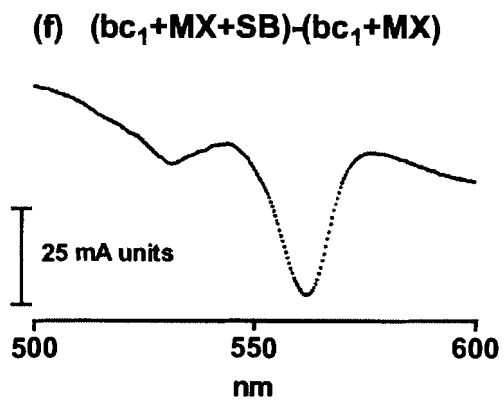
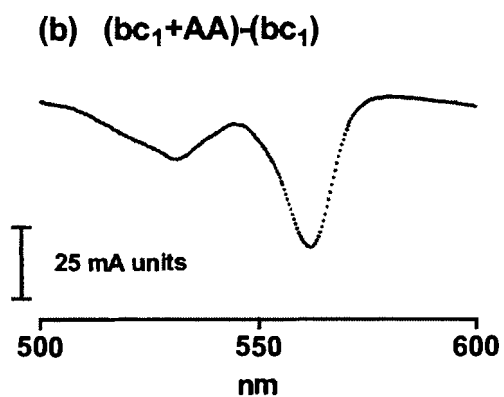
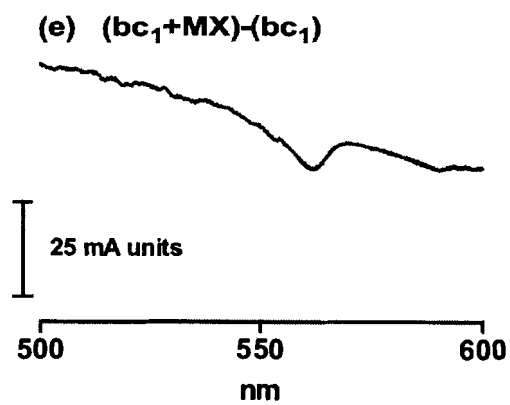
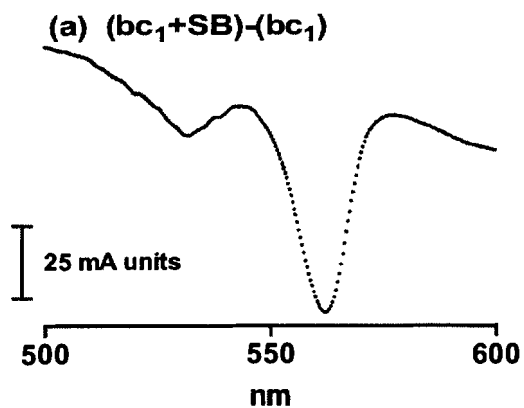


Figure 2.13 Double reciprocal plots showing the effect of increasing concentration of surangin B on complex II using different concentrations of the electron acceptor decylubiquinone compared to equivalent experiments with the inhibitors carboxin and malonate. Data points represent means \pm standard error of 3 - 6 determinations.

2.3.3 Spectral Analysis of Surangin B Binding to Purified Complex III

The spectrum of reduced complex III with surangin B bound was red shifted, indicating a trough at 558, a peak at 575 and was very similar to the spectrum induced by the Q_i site inhibitor antimycin (Fig. 2.14 a and b). Subtraction of the spectrum of either surangin B- or antimycin-saturated reduced complex III from the spectrum of reduced complex III equilibrated with surangin B and antimycin produced negligible change in the difference spectra (Fig. 2.14 c and d). The spectra of reduced complex III obtained in the presence of Q_o site inhibitors (myxothiazol or famoxadone) minus the spectrum of reduced complex III (Fig. 2.14 e and g), showed less similarity to spectra induced by surangin B or antimycin. Moreover, when the spectrum of reduced complex III equilibrated with either myxothiazol or famoxadone was subtracted from the spectrum of reduced complex III saturated with surangin B and the respective Q_o site inhibitor, a surangin B-like spectrum was observed in each case (Fig. 2.14 f and h).

Figure 2.14 Difference spectra obtained by scanning reduced complex III in the absence and presence of inhibitors and inhibitor combinations. (a) Spectrum of reduced complex III after equilibration with surangin B (SB; 50 μ M) minus spectrum of reduced complex III. (b) Spectrum of reduced complex III after equilibration with antimycin A (AA; 50 μ M) minus spectrum of reduced complex III. (c) Spectrum of reduced complex III after equilibration with surangin B (50 μ M) then antimycin A (50 μ M) minus spectrum of reduced complex III incubated with surangin B (50 μ M). (d) Spectrum of reduced complex III after equilibration with antimycin A (50 μ M) then surangin B (50 μ M) minus spectrum of reduced complex III incubated with antimycin A (50 μ M). (e) Spectrum of reduced complex III after equilibration with myxothiazol (MX; 50 μ M) minus spectrum of reduced complex III. (f) Spectrum of reduced complex III after equilibration with myxothiazol (50 μ M) and surangin B (50 μ M) minus spectrum of reduced complex III incubated with myxothiazol (50 μ M). (g) Spectrum of reduced complex III after equilibration with famoxadone (F; 50 μ M) minus spectrum of reduced complex III. (h) Spectrum of reduced complex III after equilibration with famoxadone (50 μ M) then surangin B (50 μ M) minus spectrum of reduced complex III incubated with famoxadone (50 μ M).



2.3.4 Functional Perturbation of Complex III by Surangin B

To determine the mechanism by which surangin B interferes with the transfer of electrons in complex III, I used 2-nitrosofluorene which stimulates cyanide-resistant oxygen consumption through creation of an electron bleed at the Q_i site linking to oxygen (Klohn et al., 1996). Mitochondria blocked at complexes I and II were allowed to respire on decylubiquinol. Preliminary experiments confirmed that under these conditions stimulation of oxygen consumption by 2-nitrosofluorene occurred in the presence of cyanide (data not shown). Inhibition of oxygen consumption by surangin B (5 μ M) or antimycin (5 μ M) was not relieved by 2-nitrosofluorene (Figs 2.15 a and b). In marked contrast, the block on decylubiquinol-driven oxygen consumption by myxothiazol (10 μ M) was overcome by 2-nitrosofluorene (Fig. 2.15 c).

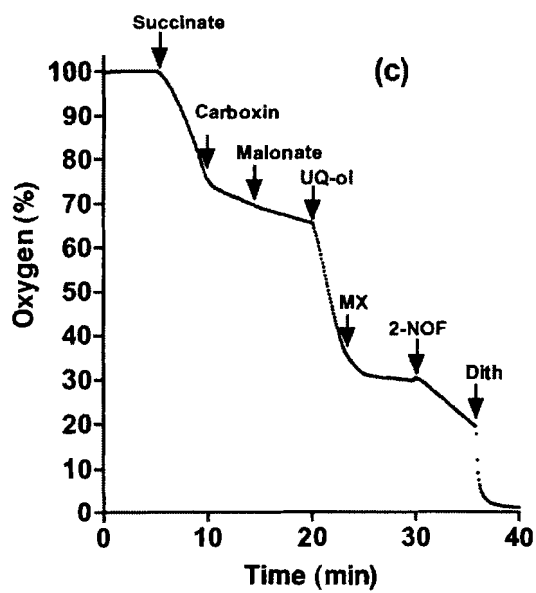
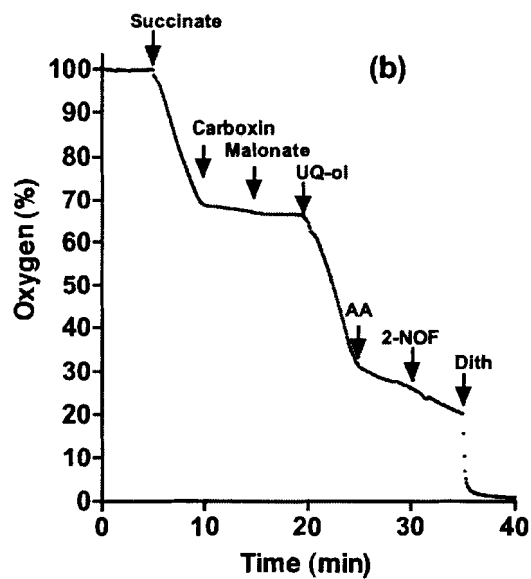
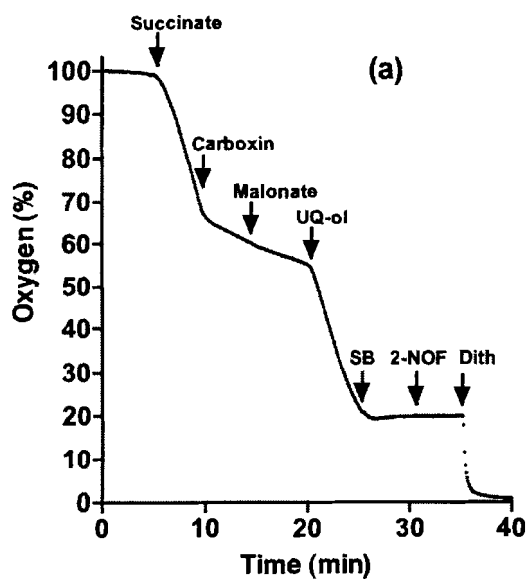
2.4 Discussion

The present investigation demonstrates that in bovine heart mitochondria the insecticidal coumarin surangin B acts as an inhibitor at three important sites regulating flow of electrons along the respiratory chain. The most sensitive site in this regard is complex II which is inhibited by 50 % with 0.2 μ M surangin B. Less potent inhibition of electron transfer through complexes III and IV was observed as evidenced by IC₅₀s of 14.8 and 3.1 μ M respectively. In addition, my results clearly show that complex I of bovine heart mitochondria is insensitive to surangin B, an observation which closely parallels the result with insect flight muscle mitochondria (Zheng et al., 1998). Also in agreement with Zheng et al. (1998) is the finding in the present investigation that

surangin B blocks complexes II and III. However, inhibition of complex IV was clearly detected in mammalian heart mitochondria, in contrast to experiments with insect mitochondria (Zheng et al., 1998). Whether this sensitivity difference to surangin B in insect and mammalian muscle reflects a species difference in the structure of complex IV remains to be demonstrated. Inhibitors which interact with more than one mitochondrial complex are not without precedent, for example the ubiquinone analog inhibitor HQNO is displaced from its binding site on complex III by antimycin (Van Ark and Berdem, 1997) and also blocks succinate:quinone oxidoreductase of *Bacillus sp.* (Qureshi et al., 1996).

The kinetic experiments involving surangin B and complex II of bovine heart mitochondria explored the way in which this coumarin might affect either the substrate (succinate) binding site or the region facilitating reduction of ubiquinone. These experiments demonstrate that the interaction of surangin B with complex II is mainly of a non-competitive type with respect to each bioenergetic intermediate. From these results, the inference can be made that surangin B does not associate directly with the binding sites of succinate or ubiquinone on complex II. Because the structure of malonate is very similar to that of succinate, malonate can competitively inhibit the binding of succinate to the enzyme. The inhibitory effect of carboxin is through binding to the membrane-anchoring proteins close to the S3 center of the Ip subunit, which blocks electron flow (Keon et al., 1994). From a kinetic perspective, this mechanism is very similar to that of the fungicide carboxin in *Ustilago maydis* (White, 1971), although the precise molecular target for surangin B on complex II remains to be identified.

Figure 2.15 Typical recordings showing the ability of 2-nitrosofluorene (2-NOF; 100 μ M) to activate decylubiquinol-driven consumption of oxygen by bovine heart mitochondria in the presence of (a) surangin B (SB; 5 μ M), (b) antimycin A (AA; 5 μ M) or (c) myxothiazol (MX; 10 μ M). Mitochondria (1.8 mg protein) and rotenone (5 μ M; to block complex I) were added before the start of recording. Succinate-stimulated electron flow through complex II was inhibited by addition of carboxin (50 μ M) and malonate (100 μ M). Following this, electron flow through complex III was accelerated by addition of decylubiquinol (UQ-ol; 150 μ M). Succinate was added at 4 mM. Dith = dithionite. The enclosed table summarizes rates before and after addition of 2-NOF. Values represent means \pm standard error of 3 - 6 separate experiments. Mean inhibitory effects of carboxin prior to UQ-ol addition for treatments a, b and c were not significantly different. Control treatments with DMSO produced no changes in O₂ consumption.



Oxygen consumption (%/min/mg)		
	Before 2-NOF	After 2-NOF
Surangin B	0.084 ± 0.027	0.023 ± 0.057
Antimycin A	0.859 ± 0.299	0.683 ± 0.186
Myxothiazol	0.082 ± 0.132	1.218 ± 0.174

Surangin B blocks artificial transfer of electrons by 2-nitrosofluorene from the Q_i site of complex III to molecular oxygen (Fig 2.16). Since I found surangin B inhibits complex IV as well as complex III, 2-nitrosofluorene offered an ideal choice for exploring interference of electron flow by surangin B at the Q_i region, because electron drain from Q_i via 2-nitrosofluorene to oxygen occurs readily when complex IV is blocked (Klohn et al., 1996). The discovery that surangin B prevents 2-nitrosofluorene from reactivating oxygen consumption in complex III provides strong evidence that this coumarin, like antimycin, interferes functionally with the Q_i site, and my results clearly distinguish surangin B mechanistically from the Q_o site inhibitor myxothiazol which the results show, in agreement with others (Klohn et al., 1996), fails to prevent this response.

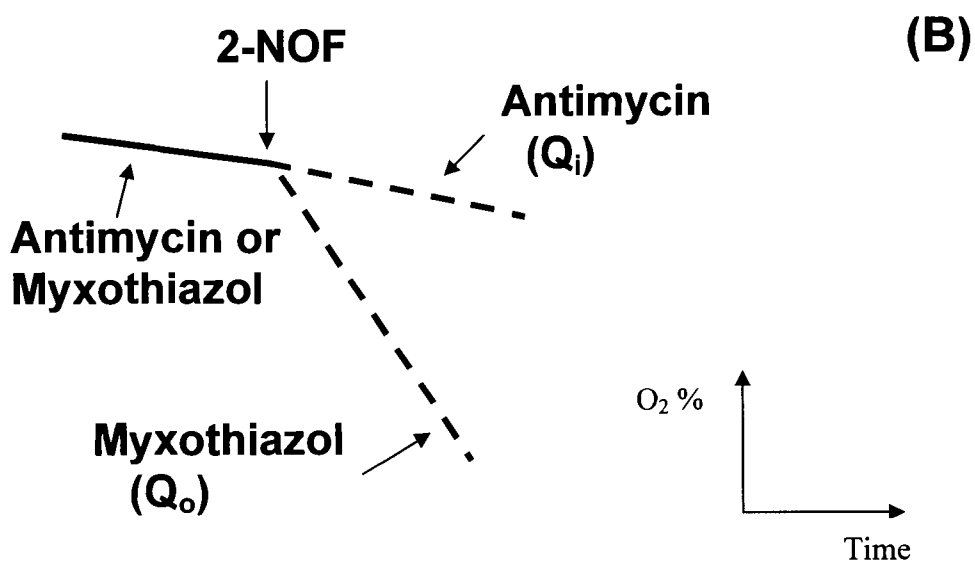
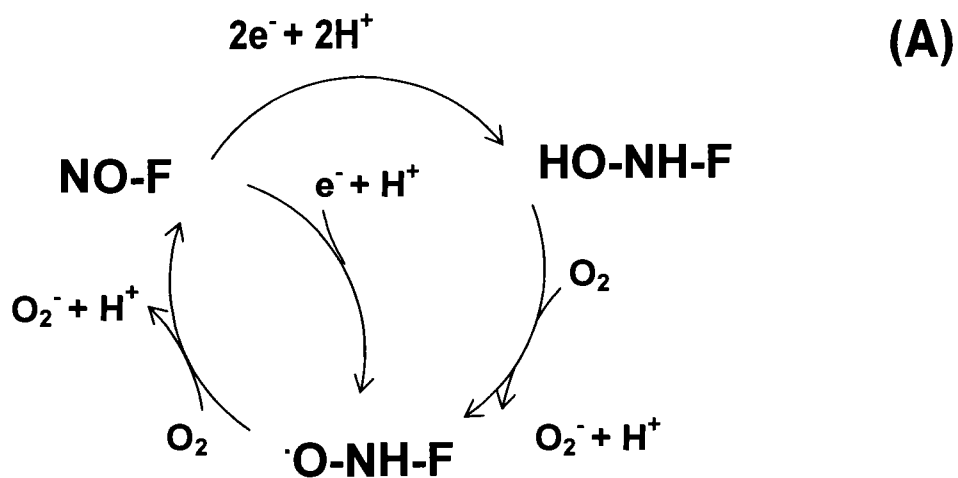


Figure 2.16 (a) Reduction of oxygen by 2-NOF as described by Klohn et al., (1995) and (b) 2-NOF effect on inhibition of complex III by antimycin and myxothiazol. Note: 2-NOF binds selectively to the Q_i site of complex III.

Equilibration of surangin B with reduced complex III induced a red shift similar to those previously observed with classical inhibitors of this complex (von Jagow and Engel, 1981; Becker et al., 1981). The difference spectra experiments on reduced complex III indicated that surangin B has the ability to bind to a site on cytochrome bc₁ that is likely associated with the antimycin A-specific Q_i binding pocket. Support for this idea is provided by the finding that the difference spectra of reduced complex III produced by surangin B and antimycin A are very similar. Moreover, when reduced complex III is first equilibrated with surangin B and then exposed to antimycin A (or *vice versa*) minimal changes in difference spectra were found. It is also apparent from these experiments that surangin B dramatically increases the difference spectrum of reduced complex III equilibrated with either myxothiazol or famoxadone, suggesting this coumarin is not targeting the Q_o pocket.

My data indicating equivalence of the difference spectra of surangin B and antimycin binding suggest that these inhibitors induce very similar conformational changes upon binding to complex III. In this respect, it can be noted that several regions of the surangin B and antimycin A molecules appear analogous (Fig 2.17). Significantly, an intramolecular hydrogen bond between the aromatic hydroxyl and carbonyl in antimycin, which is a major determinant of this antibiotic's inhibition at complex III (Miyoshi et al., 1995), is also a feature of surangin B (Crombie, 1989). This and other similarities (see Fig. 2.17) may therefore allow surangin B to bind to certain critical residues of the antimycin binding site in the Q_i pocket.

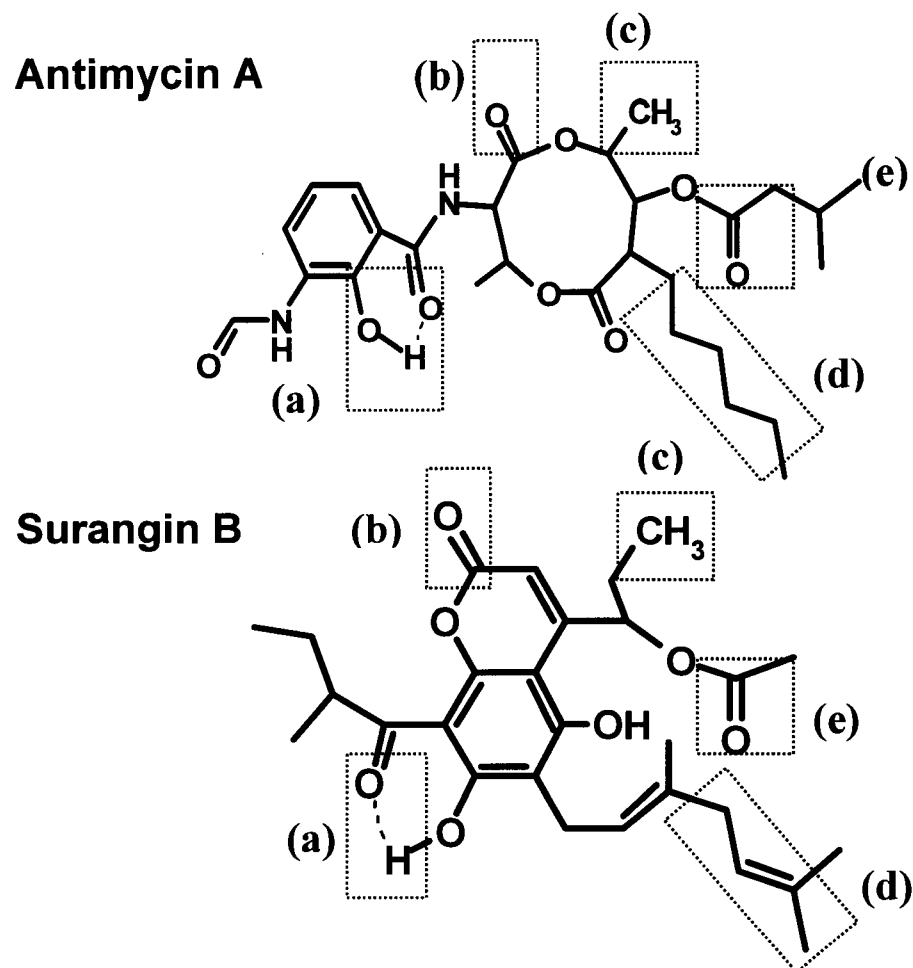


Figure 2.17 Regions of structural similarity between surangin B and antimycin A. The aromatic hydroxyl and carbonyl (a) offer opportunity for hydrogen bond bridging of surangin B to residue(s) on the Q_i binding site of complex III, as proposed previously for antimycin (Miyoshi et al., 1995). Surangin B also incorporates a lactone carbonyl (b) alkyl substituents (c and d) and an alkyl ester group (e), analogous to regions of antimycin A's dilactone ring and ring substituents which promote hydrophobic interaction of this antibiotic with the binding cavity (Miyoshi et al., 1995).

Acknowledgment

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CHAPTER 3. EFFECTS OF SURANGIN B ON THE RELEASE OF ENDOGENOUS AMINO ACIDS FROM MOUSE BRAIN SYNAPTOSOMES

3.1 Introduction

Synaptosomes are pinched-off nerve endings which have resealed, trapping their original content of cytoplasm. Synaptosomes not only retain a wide variety of functions that one would normally attribute to the nerve ending *in situ* such as synthesis, storage, release and re-uptake of transmitters but they also conserve sensitivity to the many pharmacological agents which act on these processes (Bradford, 1969; Blaustein and Goldring, 1975; Blaustein, 1975). Because synaptosomes from various brain regions have excellent biochemical and morphological preservation, they offer an invaluable system for studying neurochemical processes *in vitro* and provide highly functional preparations of nerve terminals for study (Whittaker et al., 1964; Luo and Bodnaryk, 1987). The functional integrity of the synaptosomal membrane is demonstrated by its ability to transport a variety of biomolecules including Na^+ (Ling and Abdel-Latif, 1968), K^+ (Marchbanks and Campbell, 1976; Escueta and Appel, 1969), choline (Marchbanks, 1968), GABA (Weinstein et al., 1965) and calcium (Akerman and Nicholls, 1981). Synaptosomes remain viable for several hours after isolation, during which time they display a variety cellular functions related to the processes of neurotransmission and neurosecretion (Bradford, 1975). More recently, the development of a rapid Percoll-based method to isolate highly functional synaptosomes from rat brain in high purity was reported which has the advantage that it eliminates osmotic stress during fractionation

(Dunkley et al., 1986). Consequently, in the investigations of the presynaptic actions of surangin B I describe in this thesis, I prepared synaptosomes from the brains of CD1 mice using the method of Dunkley and associates.

Separation and detection of the amino acid *o*-phthalaldehyde (OPA) derivatives can be achieved with sufficient resolution and sensitivity to follow release of glutamic acid (Glu), aspartic acid (Asp), alanine (Ala) and GABA by isolated synaptosomes. OPA-amino acids can be analyzed by high-performance liquid chromatography using a variety of systems and derivatization methods. The separation was carried out on a reversed-phase (C₁₈) column using a gradient-elution procedure.

In this chapter, I describe my investigation into the effects of surangin B on release of endogenous transmitter and non-transmitter amino acids from isolated nerve terminal preparations. The rationale for this study follows from previous observations on the neuroactive properties of surangin B (Nicholson and Zhang, 1995; Zheng et al., 1998, and has already been developed (see Insecticidal Coumarins in chapter 1.1). As hypothesized from our laboratories existing data on release of exogenously-loaded [³H]GABA and [³H]choline by synaptosomes loaded with tritiated transmitters, I found that surangin B is a potent activator of endogenous transmitter and non-transmitter amino acids (Deng and Nicholson, 2003). Moreover, my results demonstrate that surangin B releases amino acids presynaptically in a similar fashion to that of other agents which selectively target mitochondria, and that inhibition of complex III by surangin B is an important mechanism contributing to its releasing effect.

3.2 Materials and Methods

3.2.1 Chemicals

Surangin B was obtained from the roots of *M. Longifolia* as previously described (Nicholson and Zhang, 1995). Rotenone, antimycin A, carbonyl cyanide chlorophenylhydrazone (CCCP), tetramethyl-*p*-phenylenediamine (TMPD), tetrodotoxin (TTX), *o*-phthaldialdehyde and amino acid standards were obtained from Sigma-Aldrich Canada Ltd, Oakville, (ON). Carboxin and famoxadone were kindly provided by Dr. Mark Dekeyser, Uniroyal Chemical Ltd, Guelph, (ON) and Dr. Douglas Jordan, Sine Haskell Research Center, Newark, (DE), respectively.

The mobile phase was composed of phosphate buffer and methanol (HPLC grade; Rathburn, Walkersburn, Great Britain). Individual amino acid standards were obtained through Sigma. A stock solution of each amino acid was prepared by dissolving it in 10 mM HCl to provide concentration of 100 µM. Standard solutions containing 100 nM of each amino acid were prepared from the stock solution by dilution with water. All other reagents were of analytical reagent grade and used without further purification.

3.2.2 Isolation of Synaptosomes from Mouse Brain

Synaptosomes, essentially free of myelin, extraterminal (free) mitochondria and extraneous membrane fragments, were prepared from the whole brains of male CD1 mice (male, 6 - 8 weeks, 20 - 25 g) based on isoosmotic Percoll gradients according to a published method (Dunkley et al., 1986). All animal experimentation complied with the Canadian Council on Animal Care guidelines. Synaptosomes (fractions 3 and 4) were pooled, pelleted, then suspended in saline (128 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂·7H₂O, 5 mM NaHCO₃, 0.8 mM CaCl₂·2H₂O, 14 mM glucose, 20 mM HEPES;

buffered to pH 7.4 with 1.0 M Tris) to a concentration of 8 mg protein / ml and held on ice prior to assay. Protein concentration was determined using the procedure of Lowry et al., as modified by Peterson (1977).

3.2.3 Assay of Amino Acid Release from Synaptosomes

Synaptosomal suspensions (50 μ l), were added to saline (100 μ l), containing solvent control (DMSO 1 μ l), surangin B, or other compounds, as appropriate, vortexed gently, and allowed to incubate for 15 min at 32°C. After this, the mixtures were centrifuged on a Beckman Microfuge E (1 min, 4°C), and 130 μ l of each supernatant then acidified with perchloric acid (6 M; 3 μ l).

3.2.4 HPLC Analysis of Amino Acids

For high performance liquid chromatographic (HPLC) analysis (Lenda and Svenneby, 1980), samples were centrifuged again and 50 μ l of each supernatant added to borate buffer (200 μ l; 0.1 M). OPA reagent (50 μ l; Kilpatrick, 1991) to initiate derivatization and after 1 min incubation at room temperature, 40 μ l was applied to the column. OPA-amino acid separations were performed on a Hewlett Packard 1050 Chromatograph fitted with a C18 column (15 cm x 4.6 mm ID; 5 μ m particle size). The mobile phase gradient was constructed by appropriate mixing of buffer A (80 % 0.05 M sodium phosphate buffer + 20 % methanol; pH 5.7) and buffer B (20 % 0.05 M sodium phosphate buffer + 80 % methanol; pH 5.7). Elutions were initiated using buffer A (85 %) + buffer B (15 %), with programmed reduction of buffer A to 15 % over 16 min. At this stage, a 4 min elution with buffer A (85 %) + buffer B (15 %) was carried out to clean up

and re-equilibrate the column. OPA-amino acids in column eluates were analysed with an HP 1046A programmable fluorescence detector (excitation 330 nm; emission 450 nm). OPA-amino acid peak areas were quantitated and chromatographic traces recorded with an HP 3396 Series II integrator. OPA-derivatives of L-glutamic acid, GABA, aspartic acid, serine, taurine and alanine in synaptosomal supernatants were identified by comparing their retention times with standard OPA amino acids. Amino acid standards gave fluorescence responses that were linear over the working range of amino acid concentrations generated by synaptosomes in these assays. The levels of DMSO used in these assays had no effect on amino acid release from synaptosomes.

3.2.5 Analysis of Data

Curve fitting, determination of EC_{50} s with 95 % confidence limits, and statistical analyses (Student's t-test; p value < 0.05 considered significant) were carried out using Prism 3 software (GraphPad Software Inc., San Diego, CA).

3.3 Results

The relationships between concentration of surangin B and release of glutamic acid, GABA and aspartic acid from synaptosomes are given in Figures 3.1, 3.2 and 3.3 respectively, and direct comparisons with rotenone, CCCP and carboxin are also provided. Surangin B stimulated release of glutamic acid, GABA and aspartic acid in the low micromolar range as demonstrated by EC_{50} of 5.23, 11.24 and 6.59 μ M respectively (Table 3.1). Rotenone and CCCP were consistently more potent than surangin B based on

EC₅₀ which, for glutamic acid, GABA and aspartic acid, ranged from 0.19 – 0.4 μ M (rotenone) and 0.26 – 0.88 μ M (CCCP). However, at higher concentrations, surangin B released greater quantities of glutamic acid and GABA from synaptosomes than equivalent concentrations of either CCCP or rotenone. Surangin B, rotenone and CCCP also increased efflux of taurine, serine and alanine from synaptosomes (Table 3.2). By contrast, the selective complex II inhibitor carboxin was at best marginally effective at stimulating release of amino acids from synaptosomes (Figures 3.1, 3.2 and 3.3; Table 3.2). Release assays conducted in the presence of tetrodotoxin resulted in moderate reductions (glutamic acid 28 % \pm 4.8 %; GABA 34 % \pm 1.6 %; aspartic acid 33 % \pm 5.6 %; alanine 19 % \pm 0.8 %) in surangin B-evoked release of from synaptosomes (Fig 3.4), whereas this toxin had no significant inhibitory effect on surangin B-evoked release of other amino compounds (data not shown). When incubations were conducted in calcium-free incubation saline, surangin B-induced release was lower for all amino acids (Fig 3.5). Incubation of synaptosomes with TMPD, which shunts electrons around complex III (Biswas et al., 1997), inhibited surangin B-, antimycin-, and famoxadone induced release of glutamic acid (Fig 3.6), GABA (Fig 3.7), aspartic acid (Fig 3.8) and alanine (Fig 3.9).

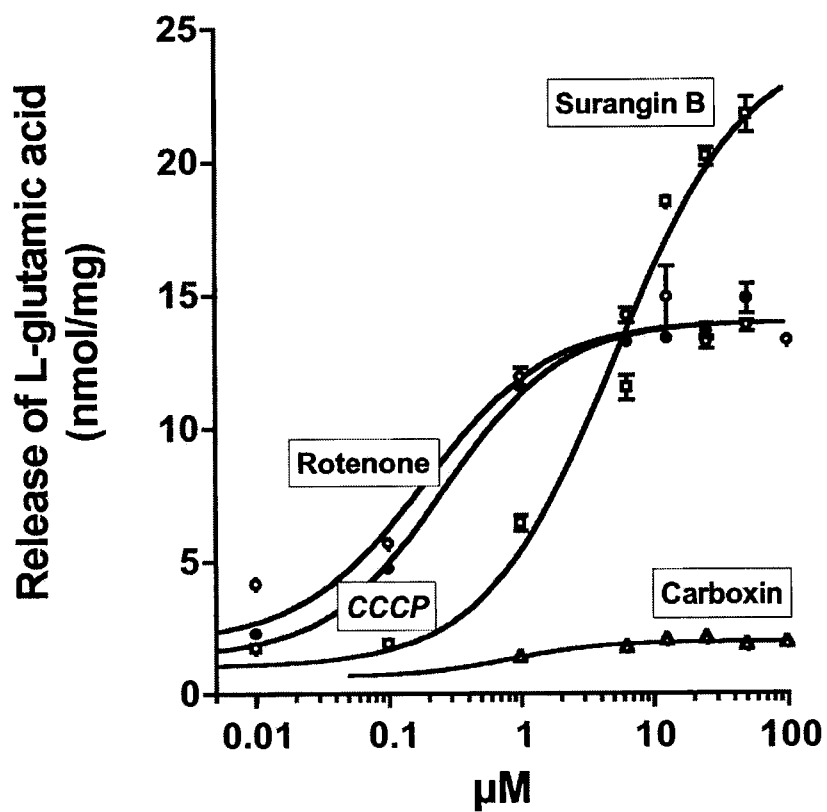


Figure 3.1 Effects of surangin B, rotenone, CCCP and carboxin on release of glutamic acid from mouse brain synaptosomes. Data points represent means \pm SE of 3 - 8 experiments.

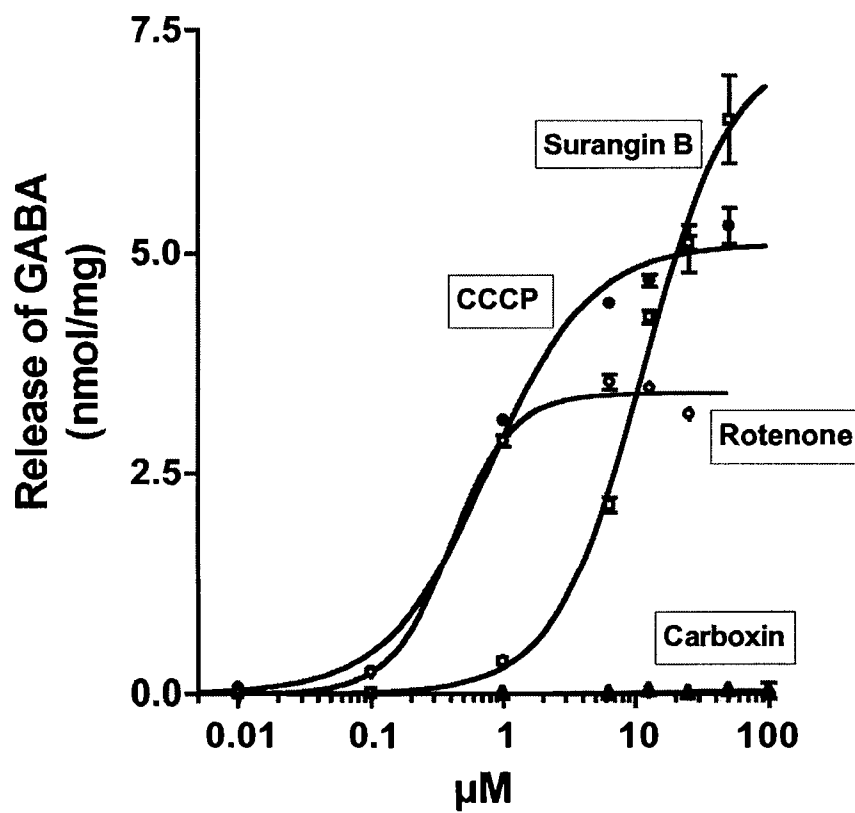


Figure 3.2 Effects of surangin B, rotenone, CCCP and carboxin on release of GABA from mouse brain synaptosomes. Data points represent means \pm SE of 3 - 8 experiments.

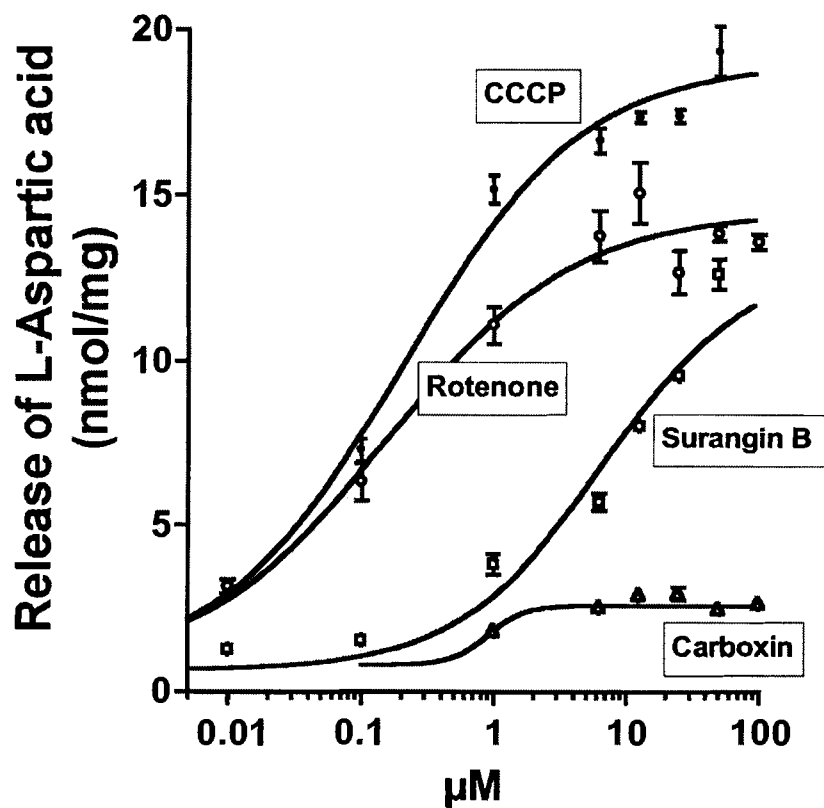


Figure 3.3 Effects of surangin B, rotenone, CCCP and carboxin on release of aspartic acid from mouse brain synaptosomes. Data points represent means \pm SE of 3 - 8 experiments.

Table 3.1 Summary of EC₅₀ data for surangin B and other compounds in relation to release of glutamic acid (GLU), γ -aminobutyric acid (GABA) and aspartic acid (ASP) from synaptosomes. Values represent EC₅₀s (μ M) and 95 % confidence limits are given in square brackets. ND = could not be accurately determined.

	Surangin B	Rotenone	CCCP	Carboxin
Glu	5.23 [3.42-7.99]	0.19 [0.09-0.39]	0.26 [0.17-0.39]	ND
GABA	11.24 [9.59- 13.18]	0.40 [0.24-0.66]	0.88 [0.58-1.34]	ND
ASP	6.59 [3.43-12.67]	0.15 [0.08-0.32]	0.20 [0.12-0.34]	ND

Table 3.2 Release of taurine (TAU), alanine (ALA) and serine (SER) by surangin B and other compounds. Values (as nmol/mg protein) represent means \pm SE of 3 - 8 experiments. (* = significantly different from control, P < 0.05).

		TAU	ALA	SER
Control		10.50 \pm 0.27	2.08 \pm 0.04	1.77 \pm 0.06
Surangin B	7 μ M	12.65 \pm 0.27	3.29 \pm 0.05	2.34 \pm 0.03
	25 μ M	12.07 \pm 0.21*	3.84 \pm 0.09*	2.40 \pm 0.06
Rotenone	7 μ M	11.94 \pm 0.05*	3.44 \pm 0.05*	2.67 \pm 0.02*
	25 μ M	11.42 \pm 0.04	3.05 \pm 0.08*	2.47 \pm 0.07
CCCP	7 μ M	11.85 \pm 0.12*	3.22 \pm 0.15*	2.73 \pm 0.30
	25 μ M	12.01 \pm 0.68	3.11 \pm 0.20	2.67 \pm 0.11*
Carboxin	25 μ M	10.24 \pm 0.08	1.89 \pm 0.05	1.85 \pm 0.06

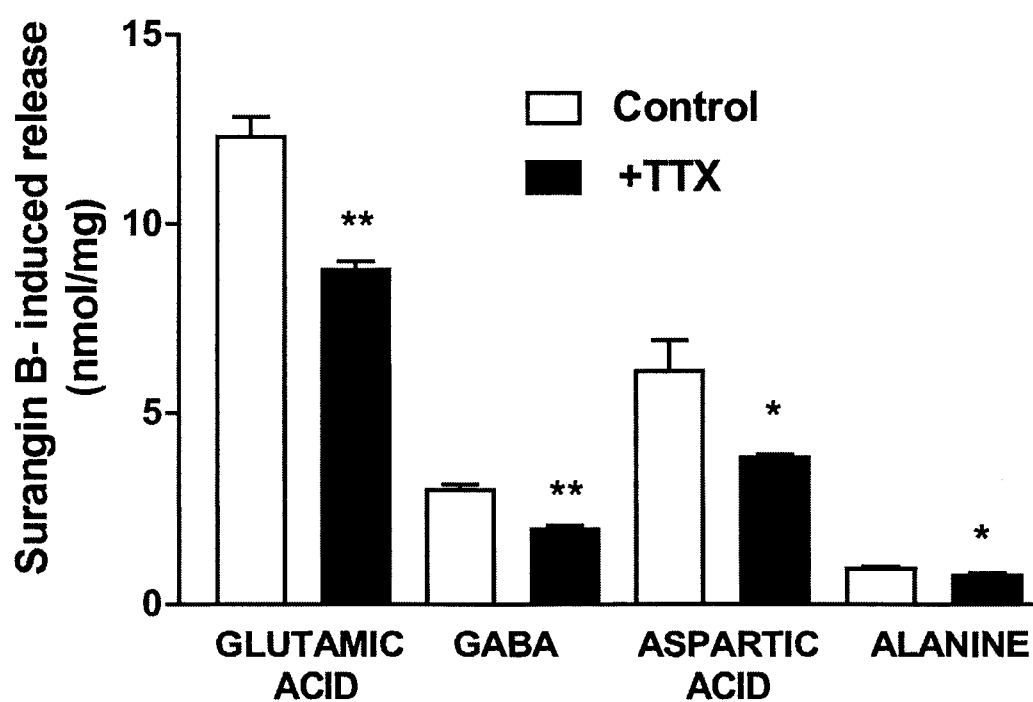


Figure 3.4 Effect of tetrodotoxin (5 μ M) on surangin B-induced release of endogenous amino acids from synaptosomes. Surangin B was applied at 7 μ M. Bars show means \pm SE of 4 experiments, (* $p < 0.05$; ** $p < 0.01$).

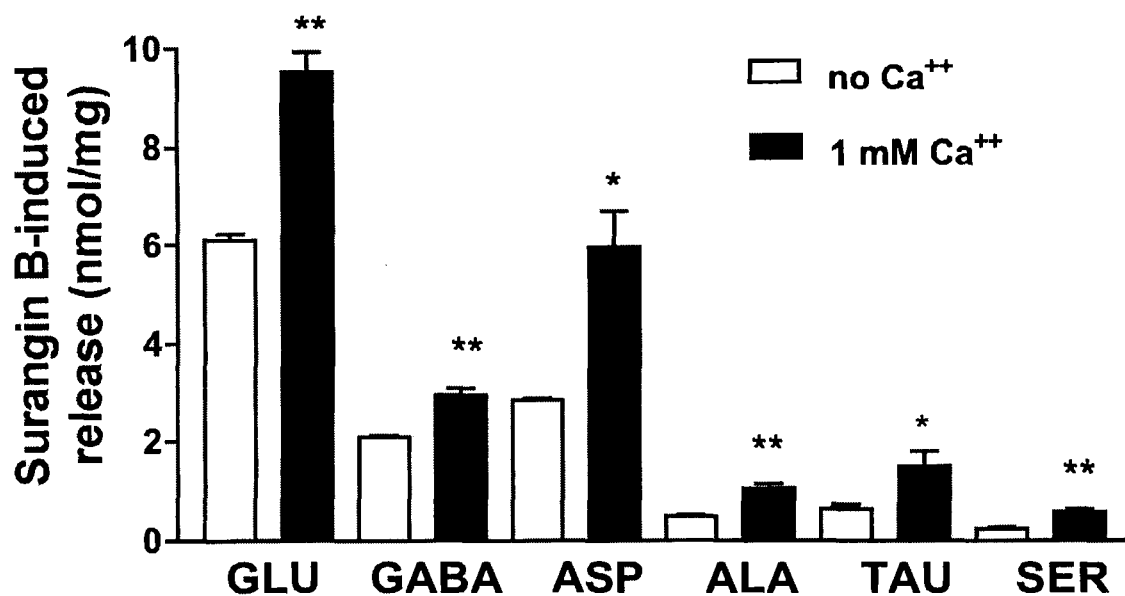


Figure 3.5 Surangin B-induced release of endogenous amino acids from synaptosomes in the absence and presence of 1 mM external calcium. Surangin B was applied at 7 μ M. Bars represent means \pm SE of 3 - 7 experiments, (* $p < 0.05$; ** $p < 0.01$). The no Ca⁺⁺ treatment contained EGTA (2 mM).

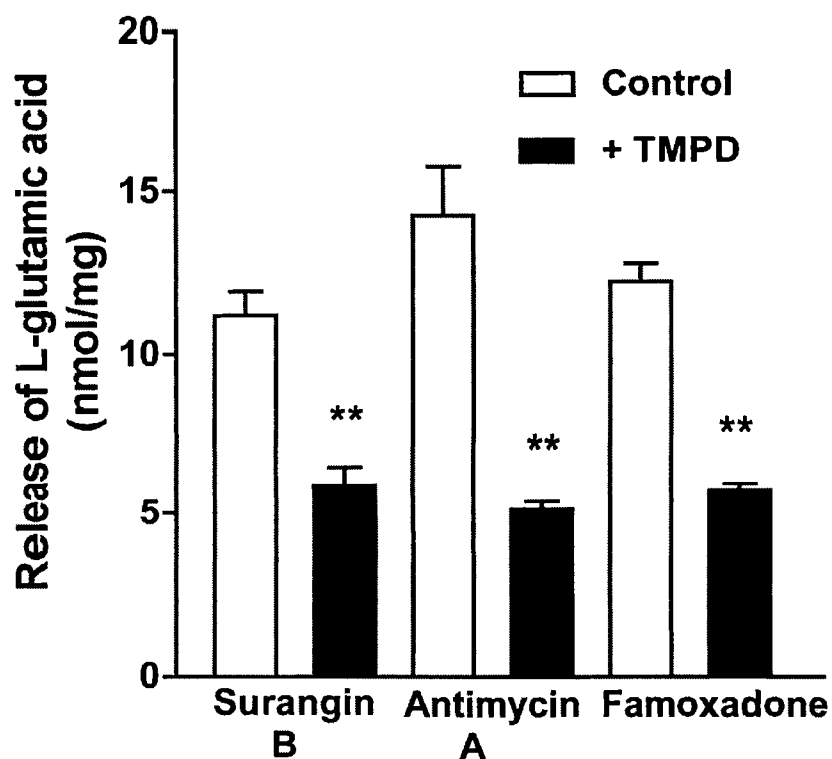


Figure 3.6 TMPD (0.5 mM) reduces the ability of surangin B, antimycin and famoxadone to release glutamic acid from synaptosomes. Surangin B and antimycin and famoxadone were applied at 7 μ M, 4 μ g/ml and 100 μ M respectively. Solid bars show stimulation by mitochondrial inhibitors above TMPD controls and data represent means \pm SE of 3 - 4 experiments, (* $p < 0.05$; ** $p < 0.01$).

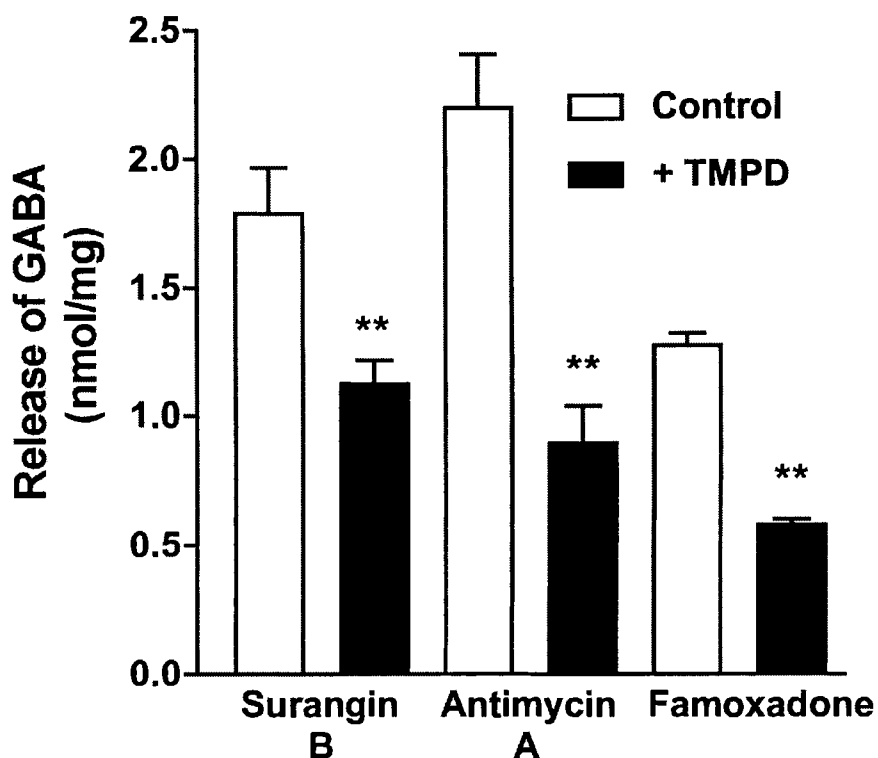


Figure 3.7 TMPD (0.5 mM) reduces the ability of surangin B, antimycin and famoxadone to release GABA from synaptosomes. Surangin B and antimycin and famoxadone were applied at 7 μ M, 4 μ g/ml and 100 μ M respectively. Solid bars show stimulation by mitochondrial inhibitors above TMPD controls and data represent means \pm SE of 3 - 4 experiments, (* $p < 0.05$; ** $p < 0.01$).

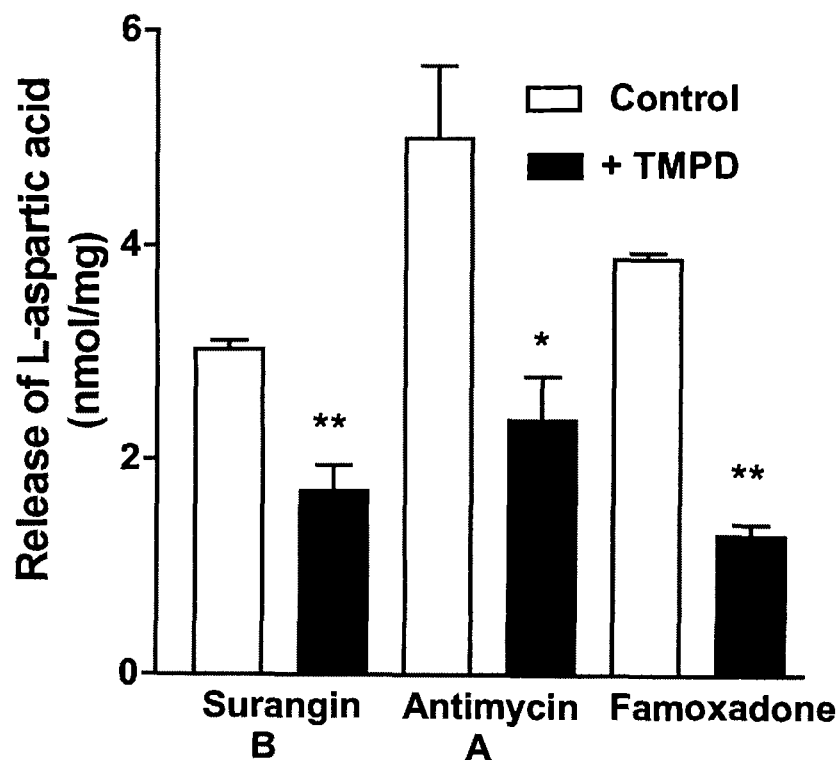


Figure 3.8 TMPD (0.5 mM) reduces the ability of surangin B, antimycin and famoxadone to release aspartic acid from synaptosomes. Surangin B and antimycin and famoxadone were applied at 7 μ M, 4 μ g/ml and 100 μ M respectively. Solid bars show stimulation by mitochondrial inhibitors above TMPD controls and data represent means \pm SE of 3 - 4 experiments, (* $p < 0.05$; ** $p < 0.01$).

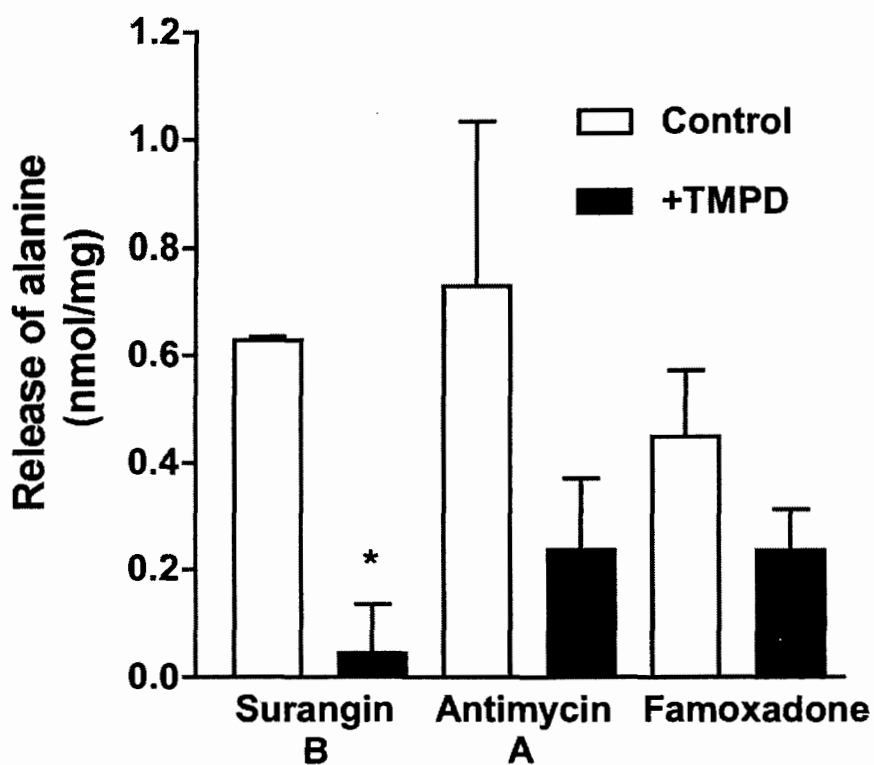


Figure 3.9 TMPD (0.5 mM) reduces the ability of surangin B, antimycin and famoxadone to release alanine from synaptosomes. Surangin B and antimycin and famoxadone were applied at 7 μ M, 4 μ g/ml and 100 μ M respectively. Solid bars show stimulation by mitochondrial inhibitors above TMPD controls and data represent means \pm SE of 3 - 4 experiments, (* $p < 0.05$; ** $p < 0.01$).

3.4 Discussion

Synaptosomes display many of the properties attributed to nerve terminals of functionally intact brain (De Belleruche and Bradford, 1973; Marchbanks and Campllell, 1976; Dodd et al., 1981). Moreover, the relative ease with which specific cellular processes involved in the release of neurotransmitters can be pharmacologically activated in synaptosomal preparations makes this preparation ideal for investigating the presynaptic actions of neuroactive compounds. Also, being virtually cellular entities, synaptosomes contain an abundance of possible neuronal sites of attack by neurotoxic chemicals. This feature clearly increases the chances of identifying novel, toxicologically relevant sites of action, so offering advantage over less complex systems.

The present investigation demonstrates that micromolar concentrations of surangin B stimulate the release of number of endogenous amino acids including the aminosulfonic acid taurine from synaptosomes isolated from mammalian brain. Within this group are substances known to have important signalling functions in brain such as the neurotransmitters glutamic acid, aspartic acid and GABA (Stephenson, 1988; Bowery, 1989; Sivilotti and Nistri, 1991; Fleck et al., 1993), the neurohumoral factor taurine (Huxtable, 1992), as well as amino acids of metabolic importance. The results therefore suggest that surangin B has potential to cause wide ranging impairment of synaptic function in mammalian brain. In previous reports, we concluded that the surangin B-induced release of radiolabel from synaptosomes preloaded with [^3H]GABA or [^3H]choline and the increase in frequency of miniature EPSCs observed in cultured cerebrocortical cells exposed to surangin B was likely a result of blockade of mitochondrial electron transport in the nerve ending (Nicholson and Zhang, 1995; Zheng

et al, 1998). The idea that interference with the bioenergetics of intraterminal mitochondrial leads to release of both neurotransmitter and metabolic amino acids from the nerve ending is strongly supported by the present results which show that rotenone, a site I inhibitor (Lindahl and Oberg, 1960; Lindahl and Oberg, 1961), CCCP which collapses the mitochondrial proton gradient (Jurkowitz et al., 1983), and antimycin and famoxadone, the latter both complex III inhibitors (Izzo et al., 1978; Jordan et al 1999a) produce effects on release which are qualitatively similar to surangin B. The validity of this type of mechanism for mitochondrial toxicants is also supported by previous work which showed that the extrasynaptosomal accumulation of glutamic acid caused by hydrogen sulphide is accompanied by depolarization of intraterminal mitochondria, inhibition of oxygen consumption and reduced ATP levels (Nicholson et al., 1998).

The ability of rotenone to cause amino acid release is consistent with mitochondria of nerve endings relying predominantly on pyruvate and other energy substrates concerned with maintaining NADH-dependent transfer of electrons to complex I of the electron transport chain. In marked contrast, my results show that carboxin, a highly potent inhibitor of mitochondrial complex II (White and Thorn, 1975; Mowery et al., 1977), was virtually inactive in causing amino acid release. This result implies that blockade of complex II in mitochondria of the nerve ending does not achieve a level of bioenergetic compromise sufficient to cause presynaptic amino acid release. The possibility that carboxin may not be able to access the mitochondrial inner membrane is unlikely since we used it at high concentrations (up to 100 μ M) and this pesticide can clearly access complex II in fungi by penetrating fungal cells.

The experiments with TMPD provide useful insight into the mechanism by which

surangin B interferes with mitochondrial function in the nerve ending. TMPD, which shunts electrons around complex III (Fig 3.10), reduced surangin B-induced release of glutamic acid, GABA, aspartic acid and alanine from synaptosomes. Furthermore, the releasing effects of other known complex III inhibitors (antimycin and famoxadone) were also reduced by TMPD to similar extents. My results therefore indicate that surangin B-induced release of amino acids from synaptosomes can arise from inhibition of complex III. It is important to note that in I found surangin B to block both complex II and complex III in bovine heart mitochondria (see Chapter 2), which implies, along with the carboxin result, that block of complex II in synaptosomes likely causes minimal release.

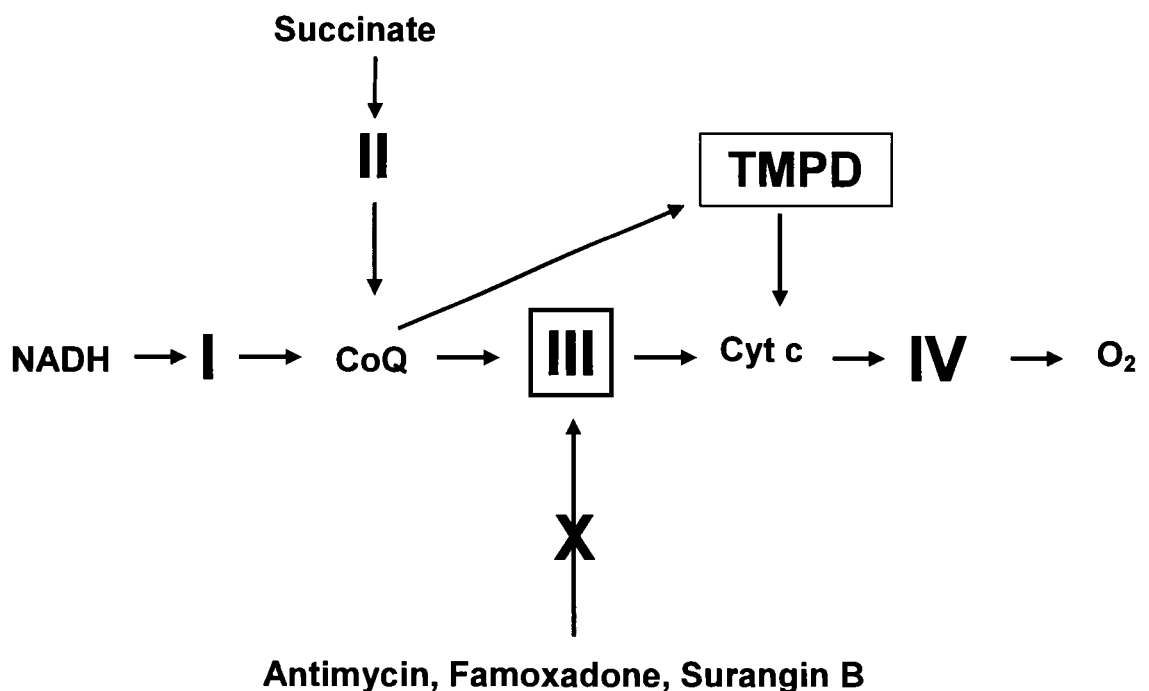


Figure 3.10 Electron transfer via TMPD.

In the present study, the extrasynaptosomal accumulation of all six amino acids

induced by surangin B was partially reduced in calcium-free saline, revealing distinct calcium-dependent and calcium-independent components to this coumarin's action. Given the mechanism I propose, one would not expect a lack of extrasynaptosomal calcium to greatly modify amino acid release arising from direct inhibition of mitochondrial function. However, the level of dependence of this response on external calcium suggests that in addition to blocking mitochondrial function, surangin B is also affecting a calcium entry mechanism to induce a moderate level of endogenous amino acid release. Reductions in amino acid efflux were also observed in the presence of tetrodotoxin (TTX), however, these inhibitory effects were less marked and were not observed for all amino acids. This result may reflect secondary activation by surangin B of voltage-gated sodium channels, since previous observations using synaptosomes (Zheng et al., 1998) show surangin B causes marked plasma membrane depolarization concurrent with mitochondrial inhibition.

In conclusion, the results of my study are consistent with the hypothesis that surangin B stimulates the release of endogenous amino acids from synaptosomes primarily through inhibition of complex III in mitochondria and to a lesser extent by stimulating calcium entry, the latter established as a mechanism for activating physiological amino acid neurotransmitter release. Impaired supply of ATP to the Na^+/K^+ ATPase can account for cyanide-induced increases in the basal efflux of glutamic acid from synaptosomes (Sanchez-Prieto et al., 1987), and ATP reduction is also associated with extrasynaptosomal glutamic acid accumulation following exposure to the cytochrome c oxidase inhibitor hydrogen sulfide (Nicholson et al., 1998). Therefore it is likely that in addition to enhanced Ca^{++} efflux from mitochondria due to failing Ca^{++} sequestration mechanisms (which will directly activate the transmitter release machinery),

bioenergetic compromise of intraterminal mitochondria by surangin B will eventually affect the energetics of plasma membrane transporters responsible for amino acid reuptake.

Acknowledgment

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CHAPTER 4. THE ANTIFUNGAL ACTIVITY OF SURANGIN B

4.1 Introduction

A variety of natural product coumarins occur as metabolic products in green plants and microorganisms (Murray, 1989; Murray, 1991; Hoult and Paya, 1996) and some of these exhibit interesting pharmacological and potentially useful therapeutic properties (Ko et al., 1989; Huang et al., 1992; Huang et al., 1993a; Itokawa et al., 1994). Coumarins present in the mature seeds of the Caribbean evergreen tree *Mammea americana* are also insecticidal (Plank, 1944; Morris and Pagan, 1953; Crombie et al., 1972; Crombie, 1989) and surangin B, a 1-(acetoxypentyl)-coumarin isolated from the roots of a related species *Mammea longifolia* of India's Western Ghats (Joshi et al., 1969), is toxic towards mosquito larvae, houseflies and crickets (Crombie et al., 1972; Crombie, 1989; Nicholson and Zhang, 1995). Surangin B has also been reported to be antibacterial (Joshi et al., 1969). In my research I have clarified the mechanisms underlying some of the the toxic actions of surangin B in muscle and brain (Deng and Nicholson, 2003; Deng and Nicholson, 2004). Together with previous work from our lab using insect and mammalian preparations (Nicholson and Zhang, 1995; Zheng et al., 1998), it is now clear that this coumarin is a potent inhibitor of mitochondrial function, blocking specifically at complexes II, III and IV, which represent critical sites regulating the flow of electrons along the respiratory chain. Inhibition of electron transport is an important mechanism by which a number of synthetic crop protection chemicals exert their fungicidal effects. For

example, in fungal and mammalian preparations, the oxathiin carboxanilide carboxin and its sulfone derivative oxycarboxin are potent inhibitors of complex II (Mowery et al., 1976; White, 1971), while famoxadone, azoxystrobin and kresoxim-methyl interfere with ubiquinone function at the Q_o site of complex III (Jordan et al., 1999a; Knight et al., 1997). My recent work has shown that like carboxin, surangin B acts as a non-competitive inhibitor of succinate and ubiquinone binding to complex II, while at complex III, surangin B mimics antimycin A by binding to the Q_i site (Deng and Nicholson, 2004). In view of the close parallels between surangin B and several fungicidal compounds in terms of the way in which they inhibit electron transport it was considered important to examine surangin B for possible *in vitro* antifungal activity.

Six species of fungi, *Alternaria dauci*, *Botrytis cinerea*, *Fusarium ocysporum*, *Penicillium* spp., *Trichoderma harzianum* and *Rhizoctonia solani*, were used in the experiments. *Botrytis cinerea* is a pathogen commonly found responsible for gray-mold rot or botrytis blight. It affects a wide range of plants including vegetable and fruit crops, as well as shrubs, trees, flowers, and weeds. *Botrytis cinerea* proliferates under cool moist conditions, so the effects of this pathogen are commonly encountered under green house conditions. *Alternaria dauci* is often found associated with plant debris in the soil and becomes readily established on the stems of seedlings below ground level, causing death. *Rhizoctonia solani* is a widely distributed soilborne pathogen which can infect a wide diversity of host plants. It mostly attacks plant parts that are below ground level such as the seeds and roots, but can also affect above ground structures such as pods, fruits, leaves and stems. *Penicillium* spp. is responsible for blue mold, which is one of the most important forms of decay of stored apples. This fungus is also known to produce

patulin, a carcinogenic mycotoxin, which is toxic to mammals. *Trichoderma harzianum* is a naturally occurring fungus that has the ability protect crops from *Botrytis*, *Rhizoctonia*, *Fusarium*, and fungi causing powdery mildew. This beneficial fungus does not appear to cause any disease or adverse health effects in humans and is not considered harmful the environment in other ways. *Trichoderma harzianum* is therefore of valuable potential in the integrated management of fungal disease.

4.2 Materials and Methods

4.2.1 Chemicals and Biological Materials

Surangin B was extracted and purified from the roots of *Mammea longifolia* according to Joshi et al., (1969). Antimycin A, potato dextrose agar and potato dextrose broth were purchased from Sigma-Aldrich Canada Ltd. Oakville (ON). I thank Dr. Douglas Jordan of the Stine Haskell Research Center, Newark, (DE) and Dr. Mark Dekeyser, Crompton Corporation, Guelph (ON) for providing the famoxadone and carboxin, respectively. All solvents and reagents employed in this study were analytical grade. The various fungal species used in this investigation (*Alternaria dauci*, *Botrytis cinerea*, *Fusarium ocysporum*, *Penicilium spp.*, *Trichoderma harzianum* and *Rhizoctonia solani*) were kindly supplied by Dr. Zamir Punja of the Department of Biological Sciences, SFU.

4.2.2 Determination of Mycelial Growth Inhibition *in vitro*

Fungal species (*Alternaria dauci*, *Botrytis cinerea*, *Fusarium oxysporum*, *Penicilium spp.*, *Trichoderma harzianum* and *Rhizoctonia solani*) were grown on potato dextrose agar on plates (60 or 90 cm) for 14 days at 22 - 24 °C. Each inoculum was prepared by blending the fungal mass complete with agar support in 50 ml of potato dextrose broth (Baya et al., 2001). The suspensions were centrifuged (Beckman RT-2000) for 1 min at 300 g to spin down the agar material. Each supernatant was then filtered through nylon fabric (100 micron mesh) and diluted to an optical density of 0.02 at 600 nm to provide the inoculum. Assays to determine inhibition of mycelial growth were carried out using 96-well plates Microtest™ tissue culture plates (Falcon, Becton Dickinson Labware) as described by Jordan et al. (1999a). To the wells of the first row was added 150 µl of fresh media while 100 µl was placed in all remaining wells. The study compound and other test substances at the highest concentration to be tested (dissolved in DMSO solvent control; 3 µl) were then added, as appropriate, to the first row of wells. After mixing thoroughly, sequential dilutions of test substances were achieved by transferring aliquots (50 µl) from the first row into the second row, mixing and then repeating down the rows. The fungal inoculum to be examined (100 µl) was then deposited in all wells, yielding a total assay volume of 200 µl and final test compound concentrations of 30, 10, 3.3, 1.1, 0.37, 0.123, 0.04 and 0.014 µM. Fungal suspensions were then incubated 24-48 hrs at 30°C. Growth inhibition was quantitated by monitoring differences in optical density (OD) at 450 nm relative to controls using a microtiter plate reader. Experiments were carried out in quadruplicate. The concentration of chemical producing 50 % inhibition of mycelial growth (IC₅₀) was estimated from

semilog concentration-inhibition curves constructed using Prism 3 software (GraphPad Software Inc., San Diego, CA).

4.2.3 Inhibition of Spore Germination *in vitro*

Fungi (*Alternaria dauci*, *Botrytis cinerea*, *Fusarium oxysporum*) were cultured on agar and allowed to sporulate. The spores were separated from the fungal mass by addition of sterile distilled water followed by agitation and the mixture was then filtered through cotton wool under sterile conditions to yield the spore suspension. The quantity of spores in suspension was determined using a hemocytometer. The germination assay was based on procedures described by others (Dhingra et al., 1995; Kim et al., 2001). Spore suspensions (1 ml) were added to eppendorf tubes containing surangin B or other inhibitors dissolved in DMSO (3 μ l), or DMSO solvent control, as appropriate, and then mixed thoroughly. Four individual 25 μ l droplets of the different spore suspensions were applied to standard microscope slides. The slides were then quickly transferred to a glass rack in a moisture chamber. The culture droplets were incubated at room temperature and then evaluated after 48 - 72 hours for the extent of spore germination. A spore was considered to have germinated if the germ tube extended at least the length of the spore. One hundred spores in three different fields were routinely counted at the light microscope level. Each experiment was repeated 3 times allowing mean percentage germination \pm standard error to be calculated. The concentration of chemical which reduced spore germination by 50 % was estimated from concentration-response curves as described in the previous section.

4.3 Results

Surangin B was an effective *in vitro* inhibitor of the mycelial growth of *Rhizoctonia solani* and *Botrytis cinerea* as evidenced by IC_{50} s of 3.8 μ M and 11.2 μ M, respectively (Fig. 4.1, Table 1). In experiments with *Rhizoctonia solani*, surangin B was comparable in potency to antimycin, but 19- and 40-fold less active than carboxin and famoxadone, respectively. Against *Botrytis cinerea*, surangin B was more potent than famoxadone, of similar activity to carboxin, but 7-fold less potent than antimycin. In contrast, surangin B was a much weaker inhibitor of mycelial growth in *Alternaria dauci*, *Fusarium oxysporum* and *Penicilium sp.*, where IC_{50} values were not achieved at 30 μ M. Interestingly, when tested for inhibition of *Fusarium oxysporum* mycelial growth, none of the other compounds had IC_{50} s below 30 μ M, although mycelial growth of *Alternaria dauci* and *Penicilium sp.* was strongly inhibited by famoxadone and antimycin (IC_{50} s = 1 μ M or below). Neither surangin B nor the synthetic fungicides were able to prevent growth of *Trichoderma harzianum* mycelium at 30 μ M and at this concentration antimycin inhibited only about 10 %. In the spore germination assays (Fig 4.2, Table 2), *Fusarium oxysporum* showed a higher sensitivity to surangin B (IC_{50} = 2.4 μ M) compared to the other inhibitors. Surangin B, famoxadone and carboxin showed similar activity in preventing germination of *Botrytis cinerea* spores (IC_{50} s = 1.4, 4.4 and 1.8 μ M respectively) but this was surpassed by antimycin (IC_{50} = 0.23 μ M). Spore germination in *Alternaria dauci* was considerably less sensitive to inhibition by surangin B (IC_{50} = 500 μ M) when compared to *Fusarium oxysporum* and *Botrytis cinerea*, however a generally weaker *in vitro* activity against *Alternaria dauci* was noted for the other test compounds.

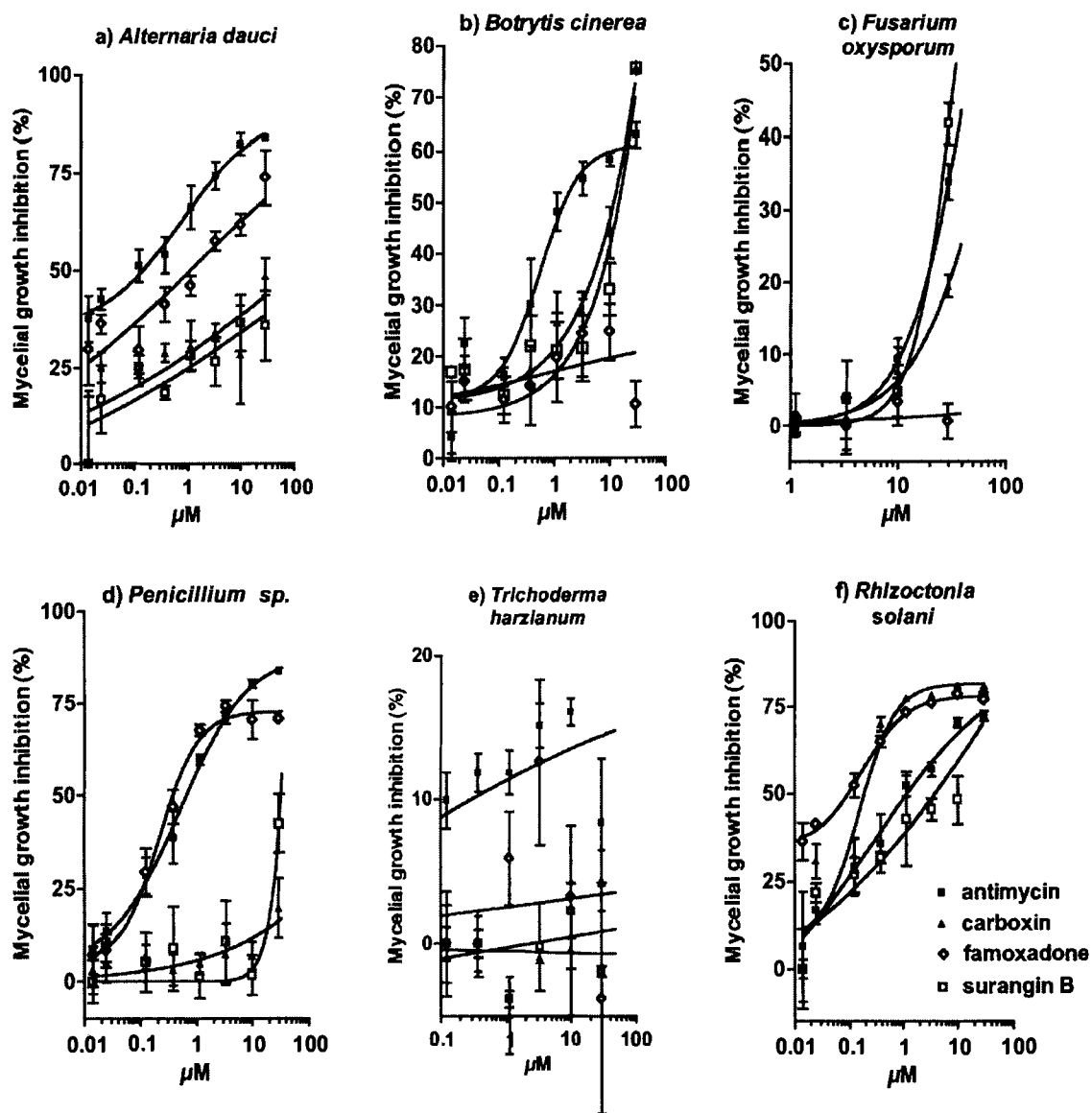


Figure 4.1 The susceptibility of the *in vitro* growth of mycelium of a) *Alternaria dauci* b) *Botrytis cinerea*, c) *Fusarium oxysporum*, d) *Penicillium sp.*, e) *Trichoderma harzianum* and f) *Rhizoctonia solani* to antimycin A, carboxin, famoxadone and surangin B. Symbols identifying all chemical treatments are given in Fig. 4.1 (f). Data points represent means \pm SEM of 4 determinations.

Table 4.1 Summary of the inhibitory effects of surangin B and other compounds on mycelial growth. Data represent IC₅₀s estimated from concentration-response curves or percentage inhibition (%) values where IC₅₀ were not achieved (see Fig. 4.1). NA = no activity detected.

	Surangin B	Carboxin	Famoxadone	Antimycin A
<i>Alternaria dauci</i>	36% at 30µM	44% at 30µM	1.2 µM	0.16 µM
<i>Botrytis cinerea</i>	11.2 µM	10.2 µM	20% at 30µM	1.6 µM
<i>Fusarium oxysporum</i>	42% at 30µM	19% at 30µM	NA	33% at 30µM
<i>Penicillium sp</i>	41% at 30µM	17% at 30µM	0.37 µM	0.63 µM
<i>Trichoderma harzianum</i>	NA	NA	NA	14% at 30µM
<i>Rhizoctonia solani</i>	3.8 µM	0.2 µM	0.09 µM	1.15 µM

4.4 Discussion

To my knowledge, this is the first report describing antifungal activity of the natural product coumarin, surangin B, from *Mammea longifolia*. In my experiments, the *in vitro* susceptibility to surangin B and the other inhibitors varied according to fungal species and whether effects on the mycelial growth or spore germination phase of the life cycle were under investigation. My results show the activity of surangin B against *Botrytis cinerea* mycelial proliferation to be similar to or better than that produced by the commercial fungicides carboxin and famoxadone, but weaker than antimycin. The other experiments on mycelial inhibition of *Rhizoctonia solani* showed that surangin B, famoxadone, carboxin and antimycin achieve similar efficacies at 30 μ M, although carboxin and famoxadone were found to be the most potent inhibitors of this group. While inhibitory effects of surangin B on spore germination in *Alternaria dauci*, *Botrytis cinerea* and *Fusarium oxysporum* were demonstrated, the sensitivity of *Fusarium oxysporum* to surangin B clearly exceeded that of the other compounds.

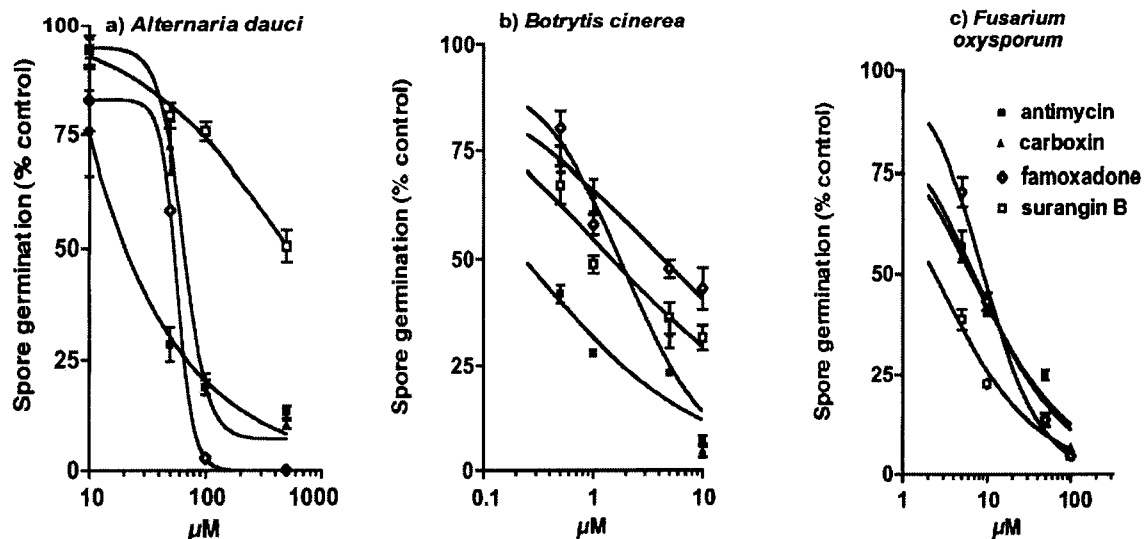


Figure 4.2 Comparison of the inhibitory effects of surangin B with carboxin, famoxadone and antimycin A on the germination of a) *Alternaria dauci* b) *Botrytis cinerea*, and c) *Fusarium oxysporum* spores *in vitro*. Symbols identifying all chemical treatments are given in Fig. 4.2 (c). Data points represent means \pm SEM of 3 determinations.

Table 4.2 Summary of the effects of surangin B and other compounds on spore germination. Values represent IC₅₀s obtained from concentration-response curves (see Fig. 4.2)

	Surangin B	Carboxin	Famoxadone	Antimycin A
<i>Alternaria dauci</i>	500 μ M	63 μ M	53 μ M	20 μ M
<i>Botrytis cinerea</i>	1.4 μ M	1.8 μ M	4.4 μ M	0.23 μ M
<i>Fusarium oxysporum</i>	2.3 μ M	6.4 μ M	9.1 μ M	6.9 μ M

My results support the idea that surangin B and indeed other coumarins of *Mammea longifolia* may have potential as prototypes for development of novel antifungals with therapeutic and/or prophylactic properties. The low activity of surangin B in some of the fungal assays may have arisen from restricted intracellular access, enhanced detoxication or reduced target site sensitivity of this coumarin. Since *Trichoderma harzianum* can be used as a fungal biocontrol agent, the insensitivity of this species to surangin B may represent an advantage in the use of coumarins in integrated fungal management programs.

CHAPTER 5. CONCLUSIONS AND FUTURE PROSPECTS

My conclusions are presented below with perspectives on future studies where I consider they are warranted.

5.1 Interaction of surangin B with bovine heart mitochondria

a) Surangin B potently blocks electron transport in bovine heart mitochondria. Complexes III and IV were inhibited at low micromolar concentrations ($IC_{50} = 14.8$ and $3.1 \mu M$ respectively), whereas complex II was the most sensitive to the inhibitory action of this coumarin ($IC_{50} = 0.2 \mu M$). Complex I of bovine heart mitochondria was unaffected by $100 \mu M$ surangin B.

b) Studies on the inhibitory effects of surangin B on the kinetics of complex II revealed that this coumarin operates as a non-competitive inhibitor of both succinate's and ubiquinone's interaction with this complex. Surangin B's inhibitory profile is clearly different from malonate (which shows competitive inhibition with succinate as substrate) but closely parallels that of the commercial fungicide carboxin. Further investigation is therefore warranted to identify the precise recognition site for surangin B on complex II. The catalytic subunits of complex II consist of a flavoprotein containing the succinate / fumarate catalytic site, and an iron sulfur complex which contains three iron-sulphur (Fe-S) centers. Electrons are transferred through complex II by a dual pathway in which electron pairs from $FADH_2$ are split. The first electron with high potential reduces Fe-S1 while the second one with low potential reduces Fe-S2. Fe-S1 then reduces Fe-S3 and Fe-

S2 reduces cytochrome b. Fe-S3 and cyt b in turn reduce ubiquinone to ubiquinol (Salerno, 1991). The Fe-S3 iron-redox center of the Ip subunit of complex II is the binding site of several inhibitors such as carboxin. A conformational change within the Fe-S3 iron-redox center is the reason site insensitivity resistance to carboxin occurs (Keon et al., 1994). Although surangin B does not show an obvious structural resemblance to carboxin (see Figs. 1.6 and 1.8), my results show that it does inhibit complex II in a very similar way. Since magnetic resonance spectroscopy has been used successfully to elucidate the site of action of inhibitors like carboxin at complex II, this approach should be advantageous in further delineating the locus of action of surangin B on this complex.

Likewise, further clarification of the binding site for surangin B on complex IV is warranted. As previously described, complex IV contains two heme groups, cytochrome α , cytochrome α_3 , and two copper ions CuA and CuB that are crucial for the transport of electrons to molecular O_2 . The binuclear center consists of an α_3 type heme of a beta form and a CuB atom of an alpha form (Pinakoulaki et al., 2002). Electron flow from cyt c following a path to CuA, then to cyt α , cyt α_3 and CuB through to molecular O_2 , with concomitant movement of H^+ from the matrix to intermembrane space. CuB is located at the O_2 delivery channel and has been proposed to play a crucial role in both the catalytic and proton pumping mechanisms of heme-copper oxidases (Pinakoulaki et al., 2004). This complex would clearly represent a critical one to investigate regarding surangin B's action. However, I feel it would be prudent to first conduct a more general assessment of potential regions of surangin B action within complex IV. Since the cytochromes of complex IV have characteristic absorption spectra in the visible region, difference spectra of the oxidized and reduced purified complex can be examined spectrophotometrically to

determine if surangin B interferes with these components. The approach would be similar to the one I used for part of the complex III study of my thesis research. Further valuable information on the site of action of surangin B on complexes II and IV would come from classical binding studies with tritiated surangin B on the purified complexes and their components. Although this would require custom radiosynthesis, it is known to that the geranyl side chain of surangin B can be reduced by hydrogen gas and the reduced form retains good electron transport inhibitory activity (Dr. Nicholson: personal communication).

c) The fact that after incubation of surangin B with purified complex III a red shift in the absorption difference spectrum is observed, provides further evidence that complex III is targeted by this coumarin. Subsequent experiments confirmed the difference spectrum of surangin B to be qualitatively and quantitatively very similar to that of antimycin A both of which are very different to those of famoxadone and myxothiazol (Q_o -site inhibitors). This result indicates that surangin B binds to the same region on complex III as antimycin A (the Q_i pocket).

d) In marked contrast to myxothiazol, the block of decylubiquinol-driven electron flow through complex III by both surangin B and antimycin A cannot be relieved by 2-NOF. Since 2-NOF specifically intercepts electrons from the Q_i -site and passes them directly to molecular oxygen, it can be concluded that surangin B cannot inhibit complex III at any location other than the Q_i -site.

e) Comparison of the structures of surangin B with antimycin A reveals several distinct regions of correspondence. I consider the aromatic hydroxyl and carbonyl group of surangin B, which provides the opportunity for hydrogen bonding between surangin B

and residues on the Q_i binding site, to be of particular significance, since such a feature has been implicated in the high affinity binding of antimycin A to Q_i . Since the topography of the Q_i pocket has now been determined it would be very informative to examine further the binding interaction between Q_i and surangin B using molecular graphic techniques.

5.2 Stimulation of endogenous amino acid release from synaptosomes by surangin B

Surangin B was found to stimulate the release of a variety of endogenous transmitter and non-transmitter amino acids from mouse brain synaptosomes at micromolar concentrations. Although similar amino acid-releasing effects were observed with rotenone and CCCP, carboxin was virtually ineffective in this regard. However, since the stimulatory effect of surangin B, antimycin and famoxadone on amino acid release was significantly reversed by TMPD, I can conclude that a substantial part of the transmitter-releasing effect of surangin B arises through block of complex III within intraterminal mitochondria. The failure of carboxin to stimulate release was unexpected, particularly since my results show this compound to be a potent inhibitor of complex II in bovine heart mitochondria. It may be that inhibition of complex II does not compromise the function of neuronal mitochondria as much as those of muscle. This hypothesis could be investigated by measuring the effect of carboxin on synaptosomal oxygen consumption. Also, other selective blockers of complex II should be evaluated in both the transmitter release and oxygen consumption assays. These latter experiments may shed light on another possibility that carboxin may not readily pass across the synaptosomal

plasma membrane to access intraterminal mitochondria. Additional experiments to find out more about the transmitter releasing action of surangin B are warranted. For example, the hypothesis that surangin B causes calcium release from intraterminal mitochondria could be tested by directly measuring release of $^{45}\text{Ca}^{++}$ from mitochondria after their isolation from mouse brain. Also, based on my experiments with Ca^{++} -free saline, calcium channel blockers, such as omega-conotoxin, should reduce surangin B-induced release. If this occurs, it will provide further evidence that some extracellular Ca^{++} must enter the nerve ending for surangin B to achieve its full effect on transmitter release. An increase in free Ca^{++} levels within the nerve ending should activate Ca^{++} CAM kinase, which will phosphorylate synapsins. This could be studied using ^{32}P labelled phosphate, followed by PAGE analysis of the phosphoproteins concerned (Zhang et al. 1996). Lastly, since botulinum toxin cleaves SNARE proteins, this toxin should totally abolish surangin B evoked transmitter release if this coumarin is acting only by activating calcium-dependent release of neurotransmitters.

5.3 Antifungal activity of surangin B

Surangin B inhibited fungal growth and spore germination in several fungal pathogens of plants but spared *Trichoderma harzanium*, a biocontrol agent. In some assays with surangin B, antifungal IC_{50}s compared favorably with those of commercial agricultural fungicides. It should be noted that only fungistatic actions of surangin B have been identified in these experiments and further work will be required to determine any fungicidal effects of this coumarin. Studies on other compounds present in the root are also warranted. My results strongly suggest that further studies should be conducted.

For example, there are at least two other coumarins (surangins A and C) present in *Mammea longifolia* and a variety of coumarins present in *Mammea americana* which should be isolated and tested for antifungal activity. During this work it will be advisable to systematically fractionate and test starting from the crude extracts, so that potential antifungal activity associated with other compounds is not missed. The range of plant fungal pathogens included in the *in vitro* tests should be extended and the *in vitro* test battery should include a range of human fungal pathogens of medical importance. Any promising activity should be followed up on an *in vivo* basis.

REFERENCE LIST

- Abrahams J. P., Leslie A. G., Lutter R. and Walker J. E. (1994). Structure at 2.8 Å resolution of F₁-ATPase from bovine heart mitochondria. *Nature* **370**, 621-628.
- Akerman K. E. and Nicholls D. G. (1981). Ca²⁺ transport by intact synaptosomes: the voltage-dependent Ca²⁺ channel and a re-evaluation of the role of sodium/calcium exchange. *Eur. J. Biochem.* **117**, 491-497.
- Alam M. and Schmidt W. J. (2002). Rotenone destroys dopaminergic neurons and induces Parkinsonian symptoms in rats. *Behav. Brain Res.* **136**, 317-324.
- Alan G. G. and Miller T. A. (1990). Long acting pyrethrin formulations. In "Pesticides and Alternatives: Innovative Chemical and Biological Approaches to Pest Control" (J.E. Casida Ed.), Elsevier Science Publishers, Amsterdam. 357-364.
- Ascher K. R. S., Klein M. and Meisner J. (1992). Azatin, a Neem formulation, acts on nymphs of the western flower thrips. *Phytoparasitica* **20**, 305-306.
- Balandrin M. F., Lee S. M. and Klocke J. A. (1988). Biologically active volatile organosulfur compounds from seeds of the neem tree, *Azadirachta indica* (Meliaceae). *J. Ag. Fd. Chem.* **36**, 1048-1054.
- Bauer R. (1997). Standardization of *Echinacea purpurea* expressed juice with reference to cichoric acid and alkamides. *Phytother.* **18**, 270- 276.
- Bauer R. and Remiger P. (1989). TLC and HPLC analysis of alkylamides in *Echinacea* drugs. *Planta Medica* **55**, 367-371.
- Baum H. and Rieske J. S. (1966). Involvement of water in electron transport in complexes III and IV of the mitochondrial electron transport chain. *Biochem. Biophys. Res. Commun.* **24**, 1-9.
- Baum H., Silman H. I., Rieske H. S. and Lipton S. H. (1967). On the composition and structural organization of Complex III of the mitochondrial electron transfer chain. *J. Biol. Chem.* **242**, 4876-4887.
- Baum H. (1967). Energetics of coupled events involving small compartments. *Nature.* **214**, 1326-1327.
- Baya M., Soullounganga P., Gelhaye E. and Gerardin P. (2001). Fungicidal activity of beta-thujaplicin analogues. *Pest Manag. Sci.* **57**, 833-838.

- Beal M. F., Brouillet E., Jenkins B., Henshaw R., Rosen B. and Hyman B. T. (1993). Age-dependent striatal excitotoxic lesions produced by the endogenous mitochondrial inhibitor malonate. *J. Neurochem.* **61**, 1147-1150.
- Beattie D. S., Patton G. M. and Stuchell R. N. (1970). Studies *in vitro* on amino acid incorporation into purified components of rat liver mitochondria. *J. Biol. Chem.* **245**, 2177-2184.
- Becker W. F., von Jagow G., Anke T. and Steglich W. (1981). Oudemansin, strobilurin A, strobilurin B and myxothiazol: New inhibitors of the *bc₁* segment of the respiratory chain with an E- β -methoxyacrylate system as a common structural element. *FEBS Letts.* **132**, 329-333.
- Bennett M. K., Calakos N. and Scheller R. H. (1991). Gap junction: new tools, new answer, new questions. *Neuron* **6**, 305-320.
- Bennett M. K. and Scheller R. H. (1993). The molecular machinery for secretion is conserved from yeast to neurons. *Proc. Natl. Acad. Sci. USA*, **90**, 2559-2563.
- Berry E. A., Huang L. and DeRose V. J. (1991). Ubiquinol-cytochrome *c* oxidoreductase of higher plants. *J. Biol. Chem.* **266**, 9064-9077.
- Betarbet R., Sherer T., MacKenzie G., Garcia-Osuna M., Panov A. V. and Greenamyre J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nature Neurosci.* **3**, 1301-1306.
- Biswas S., Ray M., Misra S., Dutta D. P. and Ray S. (1997). Selective inhibition of mitochondrial respiration and glycolysis in human leukaemic leucocytes by methylglyoxal. *Biochem. J.* **323**, 343-348.
- Blade R. J. (1989). Some aspects of synthesis and structure activity in insecticidal lipid amides. In: *Recent Advances in the Chemistry of Insect Control II*, (Ed. Crombie. L.), Royal Society of Chemistry 151-169.
- Blaustein M. P. and Goldring J. M. (1975). Membrane potentials in pinched off presynaptic nerve terminals monitored with a fluorescent probe. *J. Physiol.* **247**, 589-615.
- Blaustein M. P. (1975). Effects of potassium, veratridine and scorpion venom on calcium accumulation and transmitter release by nerve terminals *in vitro*. *J. Physiol.* **247**, 617-655.
- Bloomquist J. R., Adams P. M. and Soderlund D. M. (1986). Inhibition of gamma aminobutyric acid-stimulated chloride flux in mouse brain vesicles by polychlorocycloalkane and pyrethroid insecticides. *Neurotoxicol.* **7**, 11-20.
- Bohlmann F., Burkhardt T. and Zdero C. (1973). Naturally occurring acetylenes. Academic Press, London, New York 289-298.

- Bowery N. (1989). GABA_B receptors and their significance in mammalian pharmacology. *Trends Pharmacol. Sci.* **10**, 401-407.
- Boyer P. D. (1993). The binding change mechanism for ATP synthase: some probabilities and possibilities. *Biochim. Biophys. Acta.* **1140**, 215-250.
- Bradford H. F. (1969). Respiration *in vitro* of synaptosomes from mammalian cerebral cortex. *J. Neurochem.* **16**, 675-684.
- Bradford H. F. (1975). Isolated nerve terminals *in vitro* preparation for the study of synaptic aspects of transmitter metabolism and release. In *Handbook of Psychopharmacology; Section I: Basic Neuropharmacology*. (L.L. Iversen, S.D. Iversen, S.H. Snyder, Eds.), Plenum Press, New York **1**, 191-252.
- Brand M. D. (1985). Electroneutral efflux of Ca²⁺ from liver mitochondria. *Biochem. J.* **225**, 413-419.
- Brandt U., Haase U., Schagger H. and von Jagow G. (1991). Significance of the "Rieske" iron-sulfur protein for formation and function of the ubiquinol-oxidation pocket of mitochondrial cytochrome *c* reductase (*bc1* complex). *J. Biol. Chem.* **266**, 19958-19964.
- Brooks M. W. and Clark J. M. (1987) Enhancement of norepinephrine release from rat brain synaptosomes by α -cyano-pyrethroids. *Pestic. Biochem. Physiol.* **28**, 127-139.
- Brunger A. T. (2001a). Structure of proteins involved in synaptic vesicle fusion in neurons. *Annu. Rev. Biophys. Biomol. Struct.* **30**, 157-171.
- Brunger A. T. (2001b). Structural insights into the molecular mechanism of calcium-dependent vesicle-membrane fusion. *Curr. Opin. Struct. Biol.* **11**, 163-173.
- Burr S. A. and Ray D. E. (2004) Structure-activity and interaction effects of 14 different pyrethroids on voltage-gated chloride ion channels. *Toxicol. Sci.* **77**, 341-346.
- Burt P. E. and Goodchild R. E. (1971). The site of action of pyrethrin I in the nervous system of the cockroach, *Periplaneta americana*. *Entom. Exp. Appl.* **14**, 179-189.
- Burt P. E., Hart R. H., Nicholson R. A. and Wilson R. G. (1984). Mode of action of a novel insecticidal isobutylamide. *Electropharmacology of the in vitro synapse. Satellite Symposium of the 9th International Congress of Pharmacology*, St. Andrews, UK.
- Butterworth J. H. and Morgan E. D. (1986) Isolation of a substance that suppresses feeding in locusts. *J. Chem. Soc., Chem. Commun.* 23-24.

- California Department of Fish and Game (1997). Lake Davis Northern Pike Eradication Project. Final Environmental Impact Report. The Resources Agency, Sacramento, CA.
- Catterall W. A. (1980). Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.* **20**, 15-43.
- Cheng H. M., Yamamoto I. and Casida J. E. (1972). Rotenone photodecomposition. *J. Agric. Fd. Chem.* **20**, 850-856.
- Chuman H., Goto S., Karasawa M., Sasaki M., Nagashima U., Nishimura K. and Fujita T. (2000). Three-dimensional structure-activity relationships of synthetic pyrethroids: 1. Similarity in bioactive conformations and their structure-activity pattern. *Quantitative Structure-Activity Relationships* **19**, 10-21.
- Clary D. O., Griff I. C. and Rothman J. E. (1990). SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell.* **61**, 709-721.
- Cleeter M. W., Cooper J. M. and Schapira A. H. (1992). Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenyl-pyridinium: evidence for free radical involvement. *J. Neurochem.* **58**, 786-789.
- Clough J. M. and Godfrey C. R. A. (1995). Chemistry in Britain, 466-469.
- Conti F. and Weinberg R. J. (1999). Shaping excitation at glutamatergic synapses. *TINS* **22**, 451-458.
- Crombie L. (1995). Chemistry of Pyrethrins. *In* Pyrethrum Flowers- Production, Chemistry, Toxicology and Uses, Ed. J. E. Casida and G. B. Quistad. Oxford University Press, Chapter 8, 123-193.
- Crombie L., Games D. E., Haskins N. J. and Reed G. F. (1972). Extractives of *Mammea americana* L. V. The insecticidal compounds. *J Chem Soc [Perkin 1]* **18**, 2255-2260.
- Crombie L. (1989). Natural models for the design of insect control compounds: The mammeins, in: L. Crombie (Ed). *Recent Advances in the Chemistry of Insect Control II*, Royal Society of Chemistry 23-51.
- Crombie L. and Denman R. (1984). Insecticidal amides synthesis of natural of 2e-4e-10z-stereomer and related isobutylamides. *Tetrahedron Lett.* **25**, 4267-4270.
- Crombie, L. (1990). Natural models for the design of insect control compounds: the mammeins. In "Recent Advances in the Chemistry of Insect Control II". (L. Crombie ed.) , Spec. Pub. 79. The Royal Society of Chemistry, Cambridge, 23-51.

- Crompton M. and Heid I. (1978). The cycling of calcium, sodium, and protons across the inner membrane of cardiac mitochondria. *Eur. J. Biochem.* **91**, 599-608.
- Crosby D. G. (1995). Environmental fate of pyrethrins. In "Pyrethrum Flowers, Production, Chemistry, Toxicology, and Uses" (J.E. Casida and G.B. Quistad, eds), pp. 2-46. Oxford University Press. 2-46.
- Cunningham M. L., Soliman M. S., Badr M. Z. and Matthews H. B. (1995). Rotenone, an anticarcinogen, inhibits cellular proliferation but not peroxisome proliferation in mouse liver. *Cancer Lett.* **95**, 93-97.
- Curtis D. R. and Johnston G. A. R. (1974). Amino acid transmitters in the mammalian central nervous system. *Rev. Physiol.*, 1974, 69, 97-188)
- Daum G. (1985). Lipids of mitochondria. *Biochim. Biophys. Acta.* **822**, 1-42.
- De Belleruche J. S. and Bradford H. G. (1973). The synaptosome: an isolated, working, neuronal compartment. *In Progress in Neurobiology.* (G.A. Kerkut, and J.W. Phillis, Eds.), Oxford, Pergamon. **1**, 275-298.
- De Pinto V., Prezioso G. and Palmieri F. (1987). A simple and rapid method for the purification of the mitochondrial porin from mammalian tissues. *Biochim. Biophys. Acta.* **905**, 499-502.
- Deecher D. C., Payne, G. T. and Soderlund, D. M. (1991) Inhibition of [³H]batrachotoxinin A 20- α -benzoate binding to mouse brain sodium channels by the dihydropyrazole insecticide RH 3421. *Pestic. Biochem. Physiol.* **41**, 265-273.
- Deng Y. and Nicholson R. A. (2003). Stimulation by surangin B of endogenous amino acid release from synaptosomes. *Toxicon* **42**, 351-357.
- Deng Y. and Nicholson R. A. (2004). Block of electron transport by surangin B in bovine heart mitochondria. *Pestic. Biochem. Physiol.* In press.
- Desai V. G., Feuers R. J., Hart R. W. and Ali S. F. (1996). MPP(+)-induced neurotoxicity in mouse is age-dependent, evidenced by the selective inhibition of complexes of electron transport. *Brain Res.* **715**, 1-8.
- Ding H., Robertson D. E., Daldal F. and Dutton P. L. (1992). Cytochrome *bc1* complex [2Fe-2S] cluster and its interaction with ubiquinone and ubihydroquinone at the Qo site: a double-occupancy Qo site model. *Biochem.* **31**, 3144-3158.
- Dhingra O. D. and Sinclair J. B. Basic plant pathology methods. (1995). CRC press Inc., 227-228
- Dodd P. R., Hardy J. A., Oakles A. E., Edwardson J. A., Perry E. K. and Delaunoy J. P. (1981). A rapid method for preparing synaptosomes: comparison with alternative procedures. *Brain Res.* **226**, 107-118.

- Duce I., Warburton S., Khan, T., Thompson A. and Green C. (1999) Insect calcium channels. *In* Progress in Neuropharmacology and Neurotoxicology of Pesticides and Drugs (D.J. Beadle, Ed). SCI, Oxford, UK.
- Duke S. O. (1990). Natural Pesticides from Plants. *In* J. Janick and J.E. Simon (Eds.), Advances in new crops. Timber Press, Portland, OR 511-517.
- Dullenkopf A. and Borgeat A. (2003). Local anesthetics. Differences and similarities in the "-cains". *Anaesthesist*. **52**, 329-40.
- Dunkley P. R., Jarvie P. E., Heath J. W., Kidd G. J. and Rostas J. A. P. (1986). A rapid method for isolation of synaptosomes on percoll gradients. *Brain Res.* **372**, 115-129.
- Elliott M. (1995). Chemicals in Insect Control *In* "Pyrethrum Flowers, Production, Chemistry, Toxicology, and Uses" (J.E. Casida and G.B. Quistad, eds). Oxford University Press. 3-31.
- Elliott M., Farnham A., Janes N. F., Johnson D. M. and Pulman D. A. (1987a) Synthesis and insecticidal activity of lipophilic amides. Part 1: Introductory survey and discovery of an active synthetic compound. *Pestic. Sci.* **18**, 191-201.
- Elliott M., Farnham A., Janes N. F., Johnson D. M. and Pulman D. A. (1987b) Synthesis and insecticidal activity of lipophilic amides. Part 2: Effect of heteroatom replacements and of introducing methyl groups in the pentadiene system. *Pestic. Sci.* **18**, 203-209.
- Elliott M. and Janes N. J. (1973). Chemistry of the natural pyrethrins. *In* "Pyrethrum, The Natural Product Insecticide" (J. E. Casida, Ed.). Academic Press, New York. 55-100.
- Escueta A. V. and Appel S. H. (1969). Biochemical studies of synapses *in vitro*. II. Potassium transport. *Biochemistry* **8**, 725-733.
- Fang N. and Casida J. E. (1998). Anticancer action of cube insecticide: Correlation for rotenoid constituents between inhibition of NADH:ubiquinone oxidoreductase and induced ornithine decarboxylase activities. *Proc. Natnl. Acad. Sci. USA* **95**, 3380-3384.
- Fiebig K. M., Rice L. M., Pollock E. and Brunger A. T. (1999). Folding intermediates of SNARE complex assembly. *Nat. Struct. Biol.* **6**, 117-123.
- Fleck M. W., Henze D. A., Barrionuevo G. and Palmer A. M. (1993). Aspartate and glutamate mediate excitatory synaptic transmission in area CA1 of the hippocampus. *J. Neurosci.* **13**, 3944-3955.

- Fukami H. and Nakajima M. (1971). Rotenone and Rotenoids. In Naturally Occurring Insecticides. Eds. Jacobson, M. and Crosby, D.G. (Marcel Dekker, New York), 71-98.
- Fukami J., Shishido T., Fukunaga K. and Casida J. E. (1969). Oxidative metabolism of rotenone in mammals, fish and insects. *J. Agric. Fd. Chem.* **17**, 1217-1226.
- Fukami J. I. (1976). In *Insect Biochemistry and Physiology*. Ed. Wilkinson, C.F. (Plenum, New York) 353-396.
- Gao X., Wen X., Yu C., Esser L., Tsao S., Quinn B., Zhang L., Yu L. and Xia D. (2002). The crystal structure of mitochondrial cytochrome *bc1* in complex with famoxadone: the role of aromatic-aromatic interaction in inhibition. *Biochem.* **41**, 11692-11702.
- George C. L. and Ferguson S. J. (1987). Immunochemical probing of the structure and cofactor of NADH dehydrogenase from *Paracoccus denitrificans*. *Biochem. J.* **244**, 661-8.
- Gerberg E. J. (1995). Pyrethrum for control of pests of medical and veterinary importance. In "Pyrethrum Flowers, Production, Chemistry, Toxicology, and Uses" (J.E.Casida and G.B. Quistad, Eds). Oxford University Press. 302-309.
- Gerhauser C., Kosmeder J. W., Lee S. K., Mar W., Moriarty R. M. and Pezzuto J. M. (1996). Rotenoids as cancer chemopreventive agents: Investigation of mode of action. *Am. Chem. Soc. Abstr.* **211**, MEDI 81.
- Gerth K., Irschik H., Reichenbach H. and Trowitzsch W. (1980). Myxothiazol, an antibiotic from *Myxococcus fulvus* (myxobacterales). I. Cultivation, isolation, physico-chemical and biological properties. *J. Antibiot.* **33**, 1474-1479.
- Gilkerson R. W., Selker J. M. and Capaldi R. A. (2003). The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. *FEBS Lett.* **546**, 355-358.
- Glaser E. and Norling B. (1983). Kinetics of interaction between the H⁺-translocating component of the mitochondrial ATPase complex and oligomycin or dicyclohexylcarbodiimide. *Biochem. Biophys. Res. Commun.* **111**, 333-339.
- Gomez B. J. R. and Robinson N. C. (1999). Phospholipase digestion of bound cardiolipin reversibly inactivates bovine cytochrome *bc1*. *Biochem.* **38**, 9031-9038.
- Gornall A. G., Bardawill C. S. and David M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751-766.
- Gray A. J. and Soderlund D. M. (1985). Pyrethroid mammalian toxicology. In *Insecticides*, 193-248.

- Greene J. G., Porter R. H., Eller R. V. and Greenamyre J. T. (1993). Inhibition of succinate dehydrogenase by malonic acid produces an "excitotoxic" lesion in rat striatum. *J. Neurochem.* **61**, 1151-1154.
- Greengard P., Valtorta F., Czernik A. J. and Benfenati F. (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **259**, 780-785.
- Griffiths R. E. (1847) Medical botany or description of the more important plants used in medicine. Philadelphia, Lee and Blanchard, 642.
- Gudz T. I., Tserng K. Y. and Hoppel C. L. (1997). Direct inhibition of mitochondrial respiratory chain complex III by cell-permeable ceramide. *J. Biol. Chem.* **272**, 24154-24158
- Gullickson W. D. (1995). History of Pyrethrum in the 1970s and 1980s. In "Pyrethrum Flowers, Production, Chemistry, Toxicology, and Uses" (J.E. Casida and G.B. Quistad, eds). Oxford University Press. 2-46.
- Gupte S., Wu E. S., Hoechli A., Hoechli M., Jacobson K. and Sowers A. E. (1984). Relationship between lateral diffusion, collision frequency, and electron transfer of mitochondrial inner membrane oxidation-reduction components. *Proc. Natl. Acad. Sci. USA.* **81**, 2606-2610.
- Gutman M., Singer T. P., Beinert H. and Casida J. E. (1970). Reaction sites of rotenone, piericidin A, and amytal in relation to the nonheme iron components of NADH dehydrogenase. *Proc. Natl. Acad. Sci. USA.* **65**, 763-70.
- Halestrap A. P. (1978). Pyruvate and ketone-body transport across the mitochondrial membrane. Exchange properties, pH-dependence and mechanism of the carrier. *Biochem. J.* **172**, 377-387.
- Haley T. H. (1978). A review of the literature of rotenone. *J. Environ. Pathol. Toxicol.* **1**, 315-337
- Hamasur B. and Glaser E. (1992). Plant mitochondrial F_0F_1 ATP synthase. Identification of the individual subunits and properties of the purified spinach leaf mitochondrial ATP synthase. *Eur. J. Biochem.* **205**, 409-416.
- Han A. L., Yagi T. and Hatefi Y. (1988). Studies on the structure of NADH:ubiquinone oxidoreductase complex: topography of the subunits of the iron-sulfur flavoprotein component. *Arch. Biochem. Biophys.* **267**, 490-496.
- Hansen W. H., Davis K. J. and Fitzhugh O. G. (1965). Chronic toxicity of cube. *Toxicol. Appl. Pharmacol.* **7**, 535-542.
- Hatefi Y., Hanstein W. G., Galante Y. and Stiggall D. L. (1975). Mitochondrial ATP- P_i exchange complex and the site of uncoupling of oxidative phosphorylation. *Fed. Proc.* **34**, 1699-1706.

- Hatefi Y. (1985). The mitochondrial electron transport and oxidative phosphorylation system. *Ann. Rev. Biochem.* **54**, 1015-1069.
- Head S. W. (1973) Composition of pyrethrum extract and analysis of pyrethrins. *In* "Pyrethrum, The Natural Product Insecticide" (J. E. Casida, ed.) pp. 25-53. Academic Press, New York.
- Hendrick C. A. (1994). Pyrethroids. *In* "Agrochemicals from Natural Products" C.R.A. Godfrey Ed.). Marcel Dekker, Inc., New York. 63.
- Henry M. J. and Gustafson G. D. (2003). New antifungal modes of action. *In* " Chemistry of Crop Protection, Progress and Prospects in Science and Regulation" (Voss, G. and Ramos G. Eds.). Wiley, VCH 89-97.
- Henshaw R., Jenkins B. G., Schulz J. B., Ferrante R. J., Kowall N. W., Rosen B. R. and Beal M. F. (1994). Malonate produces striatal lesions by indirect NMDA receptor activation. *Brain Res.* **647**, 161-166.
- Heytler P. E. and Pritchard W. W. (1962). A new class of uncoupling agents-carbonyl cyanide phenylhydrazones. *Biochem. Biophys. Res. Comm.* **7**, 272-275.
- Hollingworth R. M. and Ahammadsahib K. I. (1995). *Rev. Pestic. Toxicol.* **3**, 277-302.
- Horga G. J. and Singer T. P. (1968). Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. *J. Biol. Chem.* **243**, 834-843.
- Hoult J. R. S. and Paya M. (1996). Pharmacological and biochemical actions of simple coumarins: Natural products with therapeutic potential. *Gen. Pharmacol.* **27**, 713-722.
- Huang H. C., Lee C. R., Weng Y. I., Lee M. C. and Lee Y. T. (1992). Vasodilator effect of scoparone (6,7-dimethoxycoumarin) from a Chinese herb. *Eur. J. Pharmacol.* **218**, 143-148.
- Huang H. C., Weng Y. I., Lee C. R., Jan T. R., Chen Y. L. and Lee Y. T. (1993a). Protection by scoparone against the alterations of plasma lipoproteins, vascular morphology and vascular reactivity in hyperlipidaemic diabetic rabbit. *Br. J. Pharmacol.* **110**, 1508-1514.
- Huang H. C., Lai M. W., Wang H. R., Chung Y. L., Hsieh L. M. and Chen C. C. (1993b). Antiproliferative effect of esculetin on vascular smooth muscle cells: possible roles of signal transduction pathways. *Eur. J. Pharmacol.* **273**, 39-44.
- Huxtable R. J. (1992). Physiological actions of taurine. *Physiol. Rev.* **72**, 101-163.
- Ikawa M., Dicke R. J., Thomas C. Allen T.C. and Karl Paul Link K. P. (1945). The principle alkaloids of sabadilla seed and their toxicity to *Musca domestica*. *J. Biol. Chem.* **159**, 517-524.

- Isman M. B., Koul O., Uczynski A. and Kaminsli J. (1990). Insecticidal and antifeedant bioactivities of neem oils and their relationship to azadirachtin content. *J. Agric. Food Chem.* **38**, 1406-1411.
- Isman M. B., Koul O., Arnason J. T., Stewart J. and Salloum G. S. (1991). Developing a neem-based insecticide for Canada. *Mem. Ent. Soc. Can.* **159**, 39-47.
- Itokawa H, Miyashita T, Morita H, Takeya K, Hirano T, Homma M, Oka K. (1994). Structural and conformational studies of [Ile7] and [Leu7]surfactins from *Bacillus subtilis* natto. *Chem. Pharm. Bull.* **42**, 604-7.
- Iwata S., Lee J. W., Okada K., Lee J. K., Iwata M., Rasmussen B., Ramaswamy S. and Jap B. K. (1998). Complete structure of the 11-subunit bovine mitochondrial cytochrome *bcl* complex. *Science* **281**, 64-71.
- Izhar K., Lior C., Nicolas G., Dalia G. and Michael G. (2004). Conversion of a scorpion toxin agonist into an antagonist highlights an acidic residue involved in voltage sensor trapping during activation of neuronal Na⁺ channels. *FASEB J.* **18**, 683-689.
- Izzo G., Guerrieri F. and Papa S. (1978). On the mechanism of inhibition of the respiratory chain by 2-heptyl-4-hydroxyquinoline-N-oxide. *FEBS Lett.* **93**, 320-322.
- Jacobson M. (1989). Pharmacology and toxicology of neem. CRC Press, Inc., Boca Raton, FL.
- Jacobson M. (1954a). Constituents of *Heliopsis* species. III. *Cis-trans* isomerism in affinin. *J. Amer. Chem. Soc.* **74**, 4606.
- Jacobson, M. (1954b) Occurrence of a pungent insecticidal principle in American coneflower roots. *Science* **120**, 125-129.
- Jacobson M. (1967). The structure of echinacein, the insecticidal component of American coneflower roots. *J. Org. Chem.* **32**, 1646-1647.
- Jacobson M. (1971). In Naturally Occuring Insecticides. Eds: Jacobson, M., Crosby, D.G., Marcel Dekker, New York 137-176.
- Javitch J. A., D'Amato R. J., Strittmatter S. M. and Snyder S. H. (1985). Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc. Natl. Acad. Sci. USA* **82**, 2173-2177.
- Jones P. S., Ley S. V., Morgan E. D. and Santafianos D. (1989). The chemistry of the neem tree. In "1988 focus on phytochemical pesticides", Volume 1, The neem tree (M. Jacobsen, Ed.). CRC Press, Boca Raton, FL, pp.19-45.

- Jordan D. B., Livingston R. S., Bisaha J. J., Duncan K. E., Rember S. O., Piccollelli M. A., Schwartz R. S., Sternberg J. A. and Tang X. S. (1999a). Mode of action of famoxadone. *Pestic. Sci.* **55**, 105-118.
- Jordan D. B., Kranis K. T., Piccollelli M. A., Schwartz R. S., Sternberg J. A. and Sun K. M. (1999b). Famoxadone and oxazolidinones: potent inhibitors of cytochrome *bcl*. *Biochem. Soc. Trans.* **27**, 577-580.
- Joshi B. S., Kamat V. N., Govindachari T. R. and Ganguly A. K. (1969). Isolation and structure of surangin A and surangin B, two new coumarins from *Mammea Longifolia* (Wight) Planch. and Triana. *Tetrahedron* **25**, 1453.
- Kandel E. R. and Siegelbaum S. (1985). Principles underlying electrical and chemical synaptic transmission. In *Principles of Neural Science*. 2nd Edition, (E.R. Kandel and J.H. Schwartz, Eds.), Elsevier, New York 89-107.
- Kelly R. B. (1988). The cell biology of the nerve terminal. *Neuron* **1**, 431-438.
- Kendig J. J. (1981). Barbiturates: active form and site of action at node of Ranvier sodium channels. *J. Pharmacol. Exp. Ther.* **218**, 175-81.
- Kennedy M. B. (1994). The biochemistry of synaptic regulation in the central nervous system. *Annu. Rev. Biochem* **63**, 571-600.
- Kennedy M. K. and Hamilton R. L. (1995). Pyrethrum for control of insects in the home. In "Pyrethrum Flowers, Production, Chemistry, Toxicology, and Uses" (J.E. Casida and G.B. Quistad, Eds). Oxford University Press. 2-46.
- Keon J. P. R., Broomfield P. L. E., White G. A. and Hargreaves J. A. (1994). A mutant form of the succinate dehydrogenase iron-sulphur protein subunit confers resistance to carboxin in the maize smut pathogen *Ustilago maydis*. *Biochem. Soc. Trans.* **22**, 234-237.
- Kilpatrick I. C. (1991). Rapid, automated HPLC analysis of neuroactive and other amino acids in microdissected brain regions and brain slice superfusates using fluorimetric detection. In: *Neuroendocrine research methods*, volume 2. Ed.: B. Greenstein. Harwood Academic Publishers, Chur, Switzerland, 555-578
- Kim J. C., Choi G.J., Park J.H., Kim H.T. and Cho K. Y. (2001). Activity against plant pathogenic fungi of phomalactone isolated from *Nigrospora sphaerica*. *Pest Manag. Sci.* **57**, 554-559.
- Klohn P. C., Massalha H. and Neumann H. G. (1995). A metabolite of carcinogenic 2-acetylaminofluorene, 2-nitrosofluorene, induces redox cycling in mitochondria. *Biochim. Biophys. Acta* **1229**, 363-72.

- Klohn P. C, Brandt U. and Neumann H. G. (1996). 2-Nitrosofluorene and N-hydroxy-2-aminofluorene react with the ubiquinone-reduction center (center N) of the mitochondrial cytochrome *bc1* complex. FEBS Lett. **389**, 233-7.
- Knight S. C., Anthony V. M., Brady A. M., Greenland A. J., Heaney S. P., Murray D. C., Powell K. A., Schulz M. A., Spinks C. A., Worthington P. A. and Youle D. (1997). Rationale and perspectives on the development of fungicides. Annu. Rev. Phytopathol. **35**, 349-372.
- Ko F. N., Wu T. S., Liou M. J., Huang T. F. and Teng C. M. (1989). Inhibition of platelet thromboxane formation and phosphoinositide breakdown by osthole from *Angelica pubescens*. Thromb. Haemost. **62**, 996-999.
- Koul O., Isman M. and Ketkar M. (1990). Properties and uses of neem, *Azadirachta indica*. Can. J. Bot. **68**, 1-11.
- Koul, O. and Isman M. B. (1991). Effects of azadirachtin on the dietary utilization and development of the variegated cutworm *Peridroma saucia*. J. Insect. Phys. **37**, 591-598.
- Krahenbuhl S., Chang M., Brass E. P. and Hoppel C. L. (1991). Decreased activities of ubiquinol:ferricytochrome c oxidoreductase (complex III) and ferrocyclochrome c: oxygen oxidoreductase (complex IV) in liver mitochondria from rats with hydroxycobalamin[c-lactam]-induced methylmalonic aciduria. J. Biol. Chem. **266**, 20998-21003.
- Krueger M. J., Singer T. P., Casida J. E. and Ramsay R. R. (1990). Evidence that the blockade of mitochondrial respiration by the neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺) involves binding at the same site as the respiratory inhibitor, rotenone. Biochem. Biophys. Res. Commun. **169**, 123-128.
- Kubo I., Klocke J. A., Matsumoto T. and Kamikawa T. (1984) Insecticidal and molluscicidal activities of isobutylamides isolated from *Fagara macrophylla* and their synthetic analogs. In "Pesticide Synthesis through Rational Approaches", American Chemical Society Symposium Series, 163-172.
- LaForge F.B., Haller H.L. and Smith L.E. (1933). The determination of the structure of rotenone. Chem. Rev. **12**, 81-184.
- LaLonde D. I. V. and Brown A. W. A. (1954). The effects of insecticides on the action potentials of insect nerve. Can. J. Zool. **32**, 74-81.
- Langston J. W., Ballard P., Tetrad J. W. and Irwin I. (1983a). Chronic Parkinsonism in humans due to a product of meperidine analog synthesis. Science **219**, 979-980.
- Langston J. W. and Ballard P. A. Jr. (1983b). Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N. Engl. J. Med. **309**, 310.

- Lees G. and Burt P. E. (1988). Neurotoxic actions of a lipid amide on the cockroach nerve cord and on locust somata maintained in short-term culture: a novel preparation for the study of Na⁺ channel pharmacology. *Pestic. Sci.* **24**, 189-191.
- Lehninger A. L. (1970). Mitochondria and calcium ion transport. *Biochem. J.* **119**, 129-138.
- Lenda K. and Svenneby G. (1980). Rapid high-performance liquid chromatographic determination of amino acids in synaptosomal extracts. *J. Chromatogr.* **198**, 516-519.
- Ley S. V., Denholm A. A., and Wood A. (1993). The Chemistry of Azadirachtin. *Nat. Prod. Rep.* **10**, Part 2, 109-157.
- Li X. Q., Hegazy M. G., Mahdi F., Jezek P., Lane R. D. and Garlid K. D. (1990). Purification of a reconstitutively active K⁺/H⁺ antiporter from rat liver mitochondria. *J. Biol. Chem.* **265**, 15316-15322.
- Li W., Shariat-Madar Z., Powers M., Sun X., Lane R.D. and Garlid K. D. (1992). Reconstitution, identification, purification, and immunological characterization of the 110-kDa Na⁺/Ca²⁺ antiporter from beef heart mitochondria. *J. Biol. Chem.* **267**, 17983-17989.
- Lin R. C. and Scheller R. H. (2000). Mechanisms of synaptic vesicle exocytosis. *Annu. Rev. Cell Dev. Biol.* **16**, 19-49.
- Lindahl P. E. and Oberg K. E. (1960). Mechanism of the physiological action of rotenone. *Nature.* **187**, 784.
- Lindahl PE and Oberg K. E. (1961). The effect of rotenone on respiration and its point of attack. *Exp. Cell Res.* **23**, 228-237.
- Ling C. M. and Abdel-Latif A. A. (1968). Studies on sodium transport in brain nerve-ending particles. *J. Neurochem.* **15**, 721-730.
- Lotlikar P. D., Miller E. C., Miller J. A. and Margreth A. (1965). The enzymatic reduction of N-hydroxyderivatives of 2-acetylaminofluorene and related carcinogens by tissue preparations. *Cancer Res.* **25**, 1743-1752.
- Lowenstein O. (1942). A method of physiological assay of pyrethrum extracts. *Nature* **150**, 760-762.
- Luo M. and Bodnaryk P. (1987). Synaptosomes and synaptosome membrane vesicles from the brain of *Mamestra configurata*: application to voltage-dependent and ATP-dependent Ca⁺⁺ ion transport studies. *Insect. Biochem.* **17**, 918.

- Luyengi L., Lee I. S., Mar W., Fong H. H. S., Pezzuto J. M. and Kinghorn A. D. (1994). Rotenoids and chalcones from *Mundulea sericea* that inhibit phorbol ester-induced ornithine decarboxylase activity. *Phytochem.* **36**, 1523-1526.
- MacLennan D. H. and Tzagoloff A. (1968). Studies on the mitochondrial adenosine triphosphatase system. IV. Purification and characterization of the oligomycin sensitivity conferring protein. *Biochem.* **7**, 1603-1610.
- Maienfisch P., Brandl F., Kobel W., Rindlisbacher A. and Senn R. (2004). A novel broad spectrum neonicotinoid insecticide. *In* Nicotinoid insecticides and the nicotinic acetylcholine receptor (Eds: Yamamoto I., Casida, J. E.), Springer 177-212.
- Maienfisch P., Angst M., Brandl F., Fischer W., Hofer D., Kayser H., Kobel W., Rindlisbacher A., Senn R., Steinemann A. and Widmer H. (2001). Chemistry and biology of thiamethoxam: A second generation neonicotinoid. *Pest Man. Sci.* **57**, 906-913.
- Maisch J. M. (1885) *Materia medica of the New Mexican Pharmacoposia*. *Am. J. Pharmacol.* **57**, 430-431.
- Malhotra V., Orci L., Glick B. S., Block M. R. and Rothman J. E. (1988). Role of an N-ethylmaleimide-sensitive transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack. *Cell* **54**, 221-227.
- Mandrik K. A., Vonsovich V. A. and Vinogradov V. V. (1983). Kinetics of the inhibition of succinate dehydrogenase in bull adrenal cortex by malonate and oxaloacetate. *Ukr Biokhim. Zh.* **55**, 503-506.
- Mannella C. A. (1982). Structure of the outer mitochondrial membrane: ordered arrays of porelike subunits in outer-membrane fractions from *Neurospora crassa* mitochondria. *J. Cell. Biol.* **94**, 680-687.
- Marchbanks R. M. (1968). The uptake of [^{14}C] choline into synaptosomes *in vitro*. *Biochem. J.* **110**, 533-541.
- Marchbanks R. M. and Campbell C. W. B. (1976). Sodium and chloride fluxes in synaptosomes *in vitro*. *J. Neurochem.* **26**, 973-980.
- Margulis and Lynn. (1981). *Symbiosis in Cell Evolution*. W.H. Freeman and Company, San Francisco, 206-227.
- Martinez S. S. and van Emden H. F. (1999). Sublethal concentrations of azadirachtin affect food intake, conversion efficiency and feeding behaviour of *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Bull. Entomol. Res.* **89**, 65-71.
- Matthysse J. G. and Schwardt H. H. (1943) Substitutes for rotenone in cattle louse control. *J. Econ. Entomol.* **36**, 768-772.

- Matsson M. and Hederstedt L. (2001). The carboxin-binding site on *Paracoccus denitrificans* succinate:quinone reductase identified by mutations J. Bioenerg. Biomembr. **33**, 99-105.
- Matsuno-Yagi A. and Hatefi Y. (1985). Studies on the mechanism of oxidative phosphorylation. Catalytic site cooperativity in ATP synthesis. J. Biol. Chem. **260**, 14424-14427.
- Matsuno-Yagi A. and Hatefi Y. (2001). Ubiquinol:cytochrome *c* oxidoreductase (complex III). Effect of inhibitors on cytochrome *b* reduction in submitochondrial particles and the role of ubiquinone in complex III. J. Biol. Chem. **276**, 19006-19011.
- McCormack J. G., Halestrap A. P. and Denton R. M. (1990). Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol. Rev. **70**, 391-425.
- Metcalf R. L and Metcalf R. A. (1993). Destructive and Useful Insects: Their Habits and Control. McGraw-Hill, New York.
- Metcalf R. L. (1955). Organic Insecticides. Interscience, New York 340-342.
- Metzler D. E. (1977). Biochemistry: The chemical reactions of living cells. Academic, New York. 525.
- Meyer U. A. and Schmid R. (1973). Hereditary hepatic porphyrias. Fed. Proc. **32**, 1649-1655.
- Michel H. (1998). The mechanism of proton pumping by cytochrome *c* oxidase. Proc. Natl. Acad. Sci. USA **95**, 12819-12824.
- Mitchell P. (1980). Protonmotive cytochrome system of mitochondria. Ann. NY Acad. Sci. **341**, 564-584.
- Miyakado M., Nakayama I., Inoue A., Ohno N. and Yoshioka H. (1982). Abstract No. IIIC-28. 5th International Congress of Pesticide Chemistry, Kyoto, Japan.
- Miyakado M., Nakayama I. and Ohno N. (1989). Insecticidal unsaturated isobutylamides. In "Insecticides of Plant Origin". J.T. Arnason, B.J.R. Philogene, and P. Morand (Eds.). American Chemical Society, 173-187.
- Miyano M. (1965). Total synthesis of rotenone. J. Am. Chem. Soc. **87**, 3958-3962.
- Mordue, A. J. and Blackwell A. (1993). Azadirachtin: an update. J. Insect. Phys. **39**, 903-924.
- Morris M. P. and Pagan C. (1953). Isolation of the toxic principles of mamey. J. Amer. Chem. Soc. **75**, 1489.

- Morton J. F. (1987). Mamey. In "Fruits of Warm Climates". (Ed. Morton, J.F.) 304-307.
- Mowery P. C., Ackrell B. A. C. and Singer T. P. (1976). Carboxins: powerful selective inhibitors of succinate oxidation in animal tissues. *Biochem. Biophys. Res. Commun.* **71**, 354-361.
- Mowery P. C., Steenkamp D. J., Ackrell A. C., Singer T. P. and White G. A. (1977). Inhibition of mammalian succinate dehydrogenase by carboxins. *Arch. Biochem. Biophys.* **178**, 495-506.
- Murayama K., Abbott N. J., Narahashi T. and Shapiro B. I. (1972). Effects of allethrin and condylactis toxin on the kinetics of sodium conductance of crayfish giant axon membranes. *Comp. Gen. Pharmacol.* **3**, 391-400.
- Murray R. D. H. (1989). Coumarins. *Nat. Prod. Report.* **8**, 591-624.
- Murray R. D. H. (1991). Coumarins, in: W. Herz, G. W. Kirby, W. Steglich and C. Tamm, (Eds.). *Progress in the Chemistry of Organic Natural Products*, Springer-Verlag, New York **58**, 84-316.
- Nalin C. M. and Cross R. L. (1982). Adenine nucleotide binding sites on beef heart F₁-ATPase. Specificity of cooperative interactions between catalytic sites. *J. Biol. Chem.* **257**, 8055-8060.
- Narahashi T. (1962). Effects of the insecticide allethrin on membrane potentials of cockroach giant axons. *J. Cell. Comp. Physiol.* **59**, 61-65.
- Narahashi T. and Anderson N. C. (1967). Mechanism of excitation block by the insecticide allethrin applied externally and internally to squid giant axons. *Toxicol. Appl. Pharmacol.* **10**, 529-547.
- Naumann K. (1990) *Synthetic Pyrethroid Insecticides: Structures and Properties*. Springer Series in Physical Environment, Springer-Verlag, January, 1990. ISBN: 3540513132
- Negherbohn W. O. (1959). *Handbook of Toxicology* (Saunders, Philadelphia). Vol.III 661-673.
- Nicholls D. G. and Akerman K. E. O. (1982). Mitochondrial Ca⁺⁺ transport. *Biochim. Biophys. Acta* **683**, 57-88.
- Nicholls D. G. and Ferguson J. S. (1992). *Bioenergetics 2*. Academic Press, Harcourt Brace Jovanovich, Publishers, 125.
- Nicholls D. G. and Crompton M. (1980). Mitochondrial calcium transport. *FEBS Lett.* **111**, 261-268.

- Nicholson R. A., Botham R. P. and Blade R. J. (1985). Interaction of sodium channel directed neurotoxins and a novel insecticidal isobutylamide with central nerve terminals prepared from the cockroach (*Periplaneta americana*). *Pestic. Sci.* **16**, 554-555.
- Nicholson R. A. and Zhang A. (1995). Surangin B: Insecticidal properties and mechanism underlying its transmitter-releasing action in nerve terminal fractions isolated from mammalian brain. *Pestic. Biochem. Physiol.* **53**, 152-163.
- Nicholson R. A., Roth S. H., Zhang A., Zheng J., Brookes J., Skrajny B. and Bennington R. (1998). Inhibition of respiratory and bioenergetic mechanisms by hydrogen sulfide in mammalian brain. *J. Toxicol. Environ. Health* **54**, 491-507.
- Nicholson R. A., Liao C., Zheng J., David L. S., Coyne L., Errington A. C., Singh G. and Lees G. (2003). Sodium channel inhibition by anandamide and synthetic cannabimimetics in brain. *Brain Res.* **978**, 194-204.
- Nobrega F. G. and Tzagoloff A. (1980). Assembly of the mitochondrial membrane system. Structure and location of the mitochondrial glutamic tRNA gene in *Saccharomyces cerevisiae*. *FEBS Lett.* **113**, 52-54.
- Ohta M., Narahashi T. and Keeler R. F. (1973). Effects of veratrum alkaloids on membrane potential and conductance of squid and crayfish giant axons. *J. Pharmacol. Exp. Ther.* **184**, 143-154.
- Ottea J. A., Payne G. T., Bloomquist J. R. and Soderlund D. M. (1989). Activation of sodium channels and inhibition of [³H]batrachotoxinin A-20- α -benzoate binding by an N-alkylamide neurotoxin. *Mol. Pharmacol.* **36**, 280-284.
- Parsons D. F. (1965). Recent advances correlating structure and function in mitochondria. *Int. Rev. Exp. Pathol.* **4**, 1-54.
- Passarella S. and Quagliariello E. (1976). The citric cycle intermediates transport in rat liver mitochondria. *Biochimie.* **58**, 989-1001.
- Perry A. S., Yamamoto I., Ishaaya I. and Perry R. Y. (1998). *Insecticides in Agriculture and Environment* (1998). Springer-Verlag, Berlin.
- Peterson G. L. (1977). A simplification of the method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83**, 346-356.
- Pickett J. A. (1988). Chemical pest control- the new philosophy. *Chem. Brit.* 137-142.
- Pickett J. A., Wadhams L. J. and Woodcock C. M. (1991). New Approaches to the development of semiochemicals for insect control. In "Proceedings of a Conference on Insect Ecology, Tabor, 1990". SPB Academic Publishing, The Hague. 333-345.

- Pinakoulaki E., Soulimane T. and Varotsis C. (2002). Fourier transform infrared (FTIR) and step-scan time-resolved FTIR spectroscopies reveal a unique active site in cytochrome *caa3* oxidase from *Thermus thermophilus*. *J. Biol. Chem.* **277**, 32867-32874.
- Pinakoulaki E., Ohta T., Soulimane T., Kitagawa T. and Varotsis C. (2004). Simultaneous resonance Raman detection of the heme *a3*-Fe-CO and CuB-CO species in CO-bound *ba3*-cytochrome *c* oxidase from *Thermus thermophilus*. Evidence for a charge transfer CuB-CO transition. *J. Biol. Chem.* **279**, 22791-22794.
- Plank H. K. (1944). Insecticidal properties of mamey and other plants in Puerto Rico. *J. Econ. Entomol.* **37**, 737-739.
- Qureshi M. H., Fujiwara T. and Fukumori Y. (1996). Succinate:quinone oxidoreductase (Complex II) containing a single heme *b* in facultative alkaliphilic *Bacillus* sp. strain YN-2000. *J. Bacteriol.* **178**, 3031-3036.
- Ragan C. I., Wilson M. T., Darley-Usmar M. and Lowe P. N. (1987). Subfractionation of mitochondria, and isolation of the protein of oxidative phosphorylation, In: V.M. Darley-Usmar, D. Rickwood and M.T. Wilson (Eds.), *Mitochondria: A practical approach*. IRL Press, London 79-112.
- Ragsdale D. S., Scheuer T. and Catterall W. A. (1991). Frequency and voltage-dependent inhibition of type IIA Na⁺ channels, expressed in a mammalian cell line, by local anesthetic, antiarrhythmic, and anticonvulsant drugs. *Mol. Pharmacol.* **40**, 756-65.
- Ramsay R. R., Krueger M. J. and Youngster S. K. (1991). Interaction of 1-methyl-4-phenyl-pyridinium ion (MPP⁺) and its analogs with the rotenone/piericidin binding site of NADH dehydrogenase. *J. Neurochem.* **56**, 1184-1190.
- Rasmussen H. (1971). Ionic and hormonal control of calcium homeostasis. *Am. J. Med.* **50**, 567-588.
- Rasmussen H. (1990). The complexities of intracellular Ca⁺⁺ signalling. *Biol. Chem. Hoppe Seyler.* **371**, 191-206.
- Ray D. E. (2004). Pesticides derived from plants and other organisms. *In Handbook of Pesticide Toxicology Volume 2*, Academic Press Inc., San Diego.
- Rembold H. (1988). The azadirachtins their structure and mode of action. *Chemical Congress of North America* **3**, AGRO 137.
- Rich P. R., Madgwick S. A., Brown S., von Jagow G. and Brandt U. (1991). MOA-stilbene: a new effector of the Q_i site of the chloroplast cytochrome *bf* complex. *Biochem. Soc. Trans.* **19**, 263S.

- Robertson D. E., Ding H., Chelminski P. R., Slaughter C., Hsu J., Moomaw C., Tokito M., Daldal F. and Dutton P. L. (1993). Hydroubiquinone-cytochrome *c*₂ oxidoreductase from *Rhodobacter capsulatus*: definition of a minimal, functional isolated preparation. *Biochem.* **32**, 1310-1317.
- Rowlands J. C. and Casida J. E. (1998). NADH: ubiquinone oxidoreductase inhibitors block induction of ornithine decarboxylase activity in MCF-7 human breast cancer cells. *Pharmacol. Toxicol.* **83**, 214-219.
- Salerno J. C. (1991). Electron transfer in succinate:ubiquinone reductase and quinol : fumarate reductase. *Biochem. Soc. Trans.* **19**(3):599-605.
- Sanchez-Prieto J., Sihra T. S. and Nicholls D. G. (1987). Characterization of the exocytotic release of glutamate from guinea-pig cerebral cortical synaptosomes. *J. Neurochem.* **49**, 58-64.
- Sandler S. R. and Karo W. (1971). Organic functional group preparations. Academic Press, New York and London **2**, 413-415.
- Santi R. and Toth C. E. (1965). Toxicology of rotenone. *Il Farmaco- Ed. Sc.* **20**, 270-279.
- Sariaslani F. S., Beale J. M. Jr, Rosazza J. P. (1984). Oxidation of rotenone by *Polyporus anceps laccase*. *J. Nat. Prod.* **47**, 692-7.
- Satoh E. and Nakazato Y. (1991) Effects of monensin and veratridine on acetylcholine release and cytosolic free Ca²⁺ levels in cerebrocortical synaptosomes of rats. *J. Neurochem.* **57**, 1270-1275.
- Schagger H. and von Jagow G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368-379.
- Scheffler E. I. (1999). Mitochondria. Published by Wiley-Liss Inc. 162.
- Schmutterer H. (1988). Potential of azadirachtin-containing pesticides for integrated pest control in developing and industrialized countries. *J. Insect Phys.* **34**, 713-719.
- Schmutterer H. (1990). Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. *Annu. Rev. Entomol.* **35**, 271-297.
- Schnaiman C. A. and Pedersen P. L. (1968). Localization of oligomycin-sensitive ADP-ATP exchange activity in rat liver mitochondria. *Biochem. Biophys. Res. Commun.* **30**, 428-433.
- Schnick R. (1974). A review of the literature on the use of rotenone in fisheries, FWS-CR-74-15, File No. PB-235 454/A1. National Technical Information Services, U.S.Department of Commerce, Sheffield, VA.

- Schoenig G. P. (1995). Mammalian toxicology of pyrethrum extract. *In* "Pyrethrum Flowers, Production, Chemistry, Toxicology, and Uses" (J.E. Casida and G.B. Quistad, Eds). Oxford University Press, 276.
- Scott I. D., Akerman K. E. and Nicholls D. G. (1980). Calcium-ion transport by intact synaptosomes. Intrasyntosomal compartmentation and the role of the mitochondrial membrane potential. *Biochem. J.* **192**, 873-880.
- Sharp R. D. (1961). New look at rotenone. *Soap and Chemical Specialities*.
- Shepherd G. (1994). Synaptic potentials and synaptic integration. *In* *Neurobiology*. 3rd edition, (G.M. Shepherd, Ed.), Oxford University Press, New York 132-150.
- Sherratt H. S. (1991). Mitochondria: structure and function. *Rev. Neurol.* **147**, 417-430.
- Silman H. I., Rieske J. S., Lipton S. H. and Baum H. (1967). A new protein component of complex III of the mitochondrial electron transfer chain. *J. Biol. Chem.* **242**, 4867-4875.
- Simpson D. P. (1967). Regulation of renal citrate metabolism by bicarbonate ion and pH: observations in tissue slices and mitochondria. *J. Clin. Invest.* **46**, 225-238.
- Singer T. P. and Ramsay R. R. (1990). Mechanism of the neurotoxicity of MPTP. *FEBS Lett.* **274**(1-2):1-8.
- Singer T. P. and Ramsay R. R. (1994). The reaction sites of rotenone and ubiquinone with mitochondrial NADH dehydrogenase. *Biochim. Biophys. Acta* **1187**, 198-202.
- Sivilotti L, Nistri A. (1991). GABA receptor mechanisms in the central nervous system. *Prog. Neurobiol.* **36**, 35-92.
- Snyder C. H., Gutierrez-Cirlos E. B. and Trumpower B. L. (2000). Evidence for a concerted mechanism of ubiquinol oxidation by the cytochrome *bc1* complex. *J. Biol. Chem.* **275**, 13535-13541.
- Soderlund D. M. (1995). Mode of action of pyrethrins and pyrethroids. *In* *Pyrethrum Flowers- Production, Chemistry, Toxicology and Uses*, Ed. J. E. Casida and G. B. Quistad. Oxford University Press, Chapter 10, 220.
- Spencer P. S., Nunn P. B., Hugon J., Ludolph A. C., Ross S. M., Roy D. N. and Robertson R. C. (1987). Guam amyotrophic lateral sclerosis, parkinsonism-dementia linked to a plant excitant neurotoxin. *Science* **237**, 517-522.
- Stephenson F. A. (1988). Understanding the GABA_A receptor: a chemically gated ion channel. *Biochem. J.* **249**, 21-32.

- Sternberg J. A., Geffken D., Adams J. B. J., Postages R., Sternberg C. G., Campbell C. L. and Moberg W. K. (2001). Famoxadone: the discovery and optimisation of a new agricultural fungicide. *Pest. Manag. Sci.* **57**, 143-152.
- Strichartz G. (1976). Molecular mechanisms of nerve block by local anesthetics. *Anesthesiol.* **45**, 421-41.
- Su H. C. F. (1985). N-Isobutylamides. *In* "Comprehensive Insect Physiology, Biochemistry and Pharmacology". Ed: Kerkut, G.A. and Gilbert, L.I. 12, 237-289, Oxford: Pergamon.
- Sudhof T. C. (2004). The synaptic vesicle cycle. *Annu. Rev. Neurosci.* **27**, 509-47.
- Sumitomo Chemical Company. Japanese Patent 212 150.
- Symington S. B., Zhang A., Karstens W., J. Van Houten J. and Clark J. M. (1999) Characterization of pyrethroid action on ciliary calcium channels in *Paramecium tetraurelia*. *Pestic. Biochem. Physiol.* **65**, 181- 193.
- Tan A. K., Ramsay R. R., Singer T. P. and Miyoshi H. (1993). Comparison of the structures of the quinone-binding sites in beef heart mitochondria. *J. Biol. Chem.* **268**, 19328-19333.
- Teng C. M., Li H. L., Wu T. S., Huang S. C., Huang T. F. (1992a). Antiplatelet actions of some coumarin compounds isolated from plant sources. *Thrombosis Res.* **66**, 549-557.
- Teng C. M., Li H. L., Yu S. M., Wu T. S., Huang S. C., Peng I. S. and Huang T. F. (1992b). Calcium channel blocking effects of three coumarin compounds isolated from citrus-grandis in rat thoracic aorta. *Asia Pacific J. Pharmacol.* **7**, 115-120.
- The Pesticide Manual (1977). Fifth Edition (Eds. H. Martin and C. R. Worthing), British Crop Protection Council.
- Thierbach G. and Reichenbach H. (1981). Myxothiazol, a new antibiotic interfering with respiration. *Antimicrob. Ag. Chemother.* **19**, 504-507.
- Tipton K. F. and Singer T. P. (1993). Advances in our understanding of the mechanisms of neurotoxicity of MPTP and related compounds. *J. Neurochem.* **61**, 1191-1206.
- Tokutake N., Miyoshi H., Nakazato H. and Iwamura H. (1993). Inhibition of electron transport of rat-liver mitochondria by synthesized antimycin A analogs. *Biochim. Biophys. Acta* **1142**, 262-268.
- Trimble W. S., Linial M. and Scheller R. H. (1991). Cellular and molecular biology of the presynaptic nerve terminal. *Annu. Rev. Neurosci.* **14**, 93-122.

- Trowitzsch W., Reifenstahl G., Wray V. and Gerth K. (1980). Myxothiazol, an antibiotic from *Myxococcus fulvus* (myxobacterales). II. structure elucidation. J. Antibiot. **33**, 1480-1490.
- Trumpower B. L. (1990a). The protonmotive Q cycle-energy transductin by coupling of proton translocation to electron transfer by the cytochrome *bc*₁ complex. J. Biol. Chem. **265**, 11409-11412.
- Trumpower B. L. (1990b). Cytochrome *bc*₁ complexes of microorganisms. Microbiol. Rev. **54**, 101-129.
- Tyler D. (1992). The mitochondrion in health & disease. VCH Publishers, Inc. 224.
- Tzagoloff A., Maclellan D. H. and Byington K. H. (1968). Studies on the mitochondrial adenosine triphosphatase system. 3. Isolation from the oligomycin-sensitive adenosine triphosphatase complex of the factors which bind F-1 and determination of oligomycin sensitivity of bound F-1. Biochem. **7**, 1596-1602.
- Ueno H., Miyoshi H., Ebisui K. and Iwamura H. (1994). Comparison of the inhibitory action of natural rotenone and its stereoisomers with various NADH-ubiquinone reductases. Eur. J. Biochem. **225**, 411-417.
- Ulbricht W. (1998). Effects of veratridine on sodium currents and fluxes. Rev. Physiol. Biochem. Pharmacol. **133**, 1-54.
- Van Ark G. and Berden J. A. (1977). Binding of HQNO to beef-heart sub-mitochondrial particles. Biochim. Biophys. Acta **459**, 119-127.
- Van Dolah F. M., Finley E. L., Haynes B. L., Doucette G. J., Moeller P. D. and Ramsdell J. S. (1994) Development of rapid and sensitive high throughput pharmacologic assays for marine phycotoxins. Nat. Toxins. **2**, 189-96.
- Verkerk R. H. J. and Wright D. J. (1993). Biological activity of neem seed kernel extracts and synthetic azadirachtin against larvae of *Plutella xylostella* L. Pestic. Sci. **37**, 83-91.
- Vijverberg H. P. M., van der Zalm J. M. and van den Bercken J. (1982). Similar mode of action of pyrethroids on sodium gating in myelinated nerves. Nature **295**, 601-603.
- von Jagow G. and Engel W. D. (1981). Complete inhibition of electron transfer from ubiquinol to cytochrome *b* by the combined action of antimycin and myxothiazol. FEBS Letters **136**, 19-24.
- Walker J. E. (1992). The NADH:ubiquinone oxidoreductase (Complex I) of respiratory chains, Q. Rev. Biophys. **25**, 253-324.

- Wang S. Y. and Wang G. K. (1998). Point mutations in segment I-s6 render voltage-gated Na⁺ channels resistant to batrachotoxin. *Proc. Natl. Acad. USA* **95**, 2653-2658.
- Wang S. Y. and Wang G. K. (2003). Voltage-gated sodium channels as primary targets of diverse lipid-soluble neurotoxins. *Cellul. Sig.* **15**, 151-159.
- Warthen J. D. (1989). Neem (*Azadirachta indica* A. Juss): organisms affected and reference list update. *Proceedings of the Entomological Society of Washington* **1**, 367-388.
- Ware G. W. (1986) *Fundamentals of Pesticides-A Self Instruction Guide*, Second edition. Thomson Publications. Fresno, CA.
- Webb J. L. (1966). *Enzyme and Metabolic Inhibitors. Volume II, Malonate analogs, dehydroacetate, sulfhydryl reagents, o-iodosobenzoate, mercurials.* New York: Academic Press.
- Weinbach E. C. and Von Brand T. (1967). Formation, isolation and composition of dense granules from mitochondria. *Biochim. Biophys. Acta.* **148**, 256-266.
- Weinstein H., Varon S., Muhleman D. R. and Roberts E. (1965). A carrier-mediated transfer mode for the accumulation of [¹⁴C] γ -aminobutyric acid by subcellular brain particles. *Biochem. Pharmacol.* **14**, 288.
- Weiss H., Friedrich T., Hofhaus G. and Preis D. (1991). The respiratory-chain NADH dehydrogenase (complex I) of mitochondria. *Eur. J. Biochem.* **197**, 563-576.
- Wellcome Foundation Ltd. (1984). Wellcome Foundation Ltd. European Patent 111 105.
- White G. A. (1971). A potent effect of 1,4-oxathiin systemic fungicides on succinate oxidation by a particulate preparation from *Ustilago maydis*. *Biochem. Biophys. Res. Commun.* **44**, 1212-1219.
- White G. A. and Thorn G. D. (1975). Structure-activity relationships of carboxamide fungicides and the succinic dehydrogenase complex of *Cryptococcus laurentii* and *Ustilago maydis*. *Pestic. Biochem. Physiol.* **5**, 380-395.
- Whittaker V. P., Michaelson I. A. and Kirkland R. J. A. (1964). The separation of synaptic vesicles from nerve-ending particles (synaptosomes). *Biochem. J.* **90**, 293-303.
- Whitton P. S., Nicholson R. A. and Strang R. H. C. (1994). Electrophysiological responses of isolated locust (*Schistocerca gregaria*) somata to taurine and GABA application. *J. Insect Physiol.* **40**, 195-199.
- Wikstrom M. K. F., Krab K. and Saraste M. (1981). *Cytochrome Oxidase: A synthesis.* New York: Academic Press.

- Wintersteiner O. (1953) The chemistry of the veratrum alkaloids. *Record of Chem. Progr.* **14**, No.1, 19-34.
- Xia D., Yu C. A., Kim H., Xia J. Z., Kachurin A. M., Zhang L., Yu L. and Deisenhofer J. (1997). Crystal structure of the cytochrome *bcl* complex from bovine heart mitochondria. *Science* **277**, 60-66.
- Zang X., Fukuda E. K. and Rosen J. D. (1997). Method for the determination of veratridine and cevadine, major components of the natural insecticide sabadilla, in lettuce and cucumbers. *J. Agri. Fd. Chem.* **45**, 1758-1761.
- Zhang A. and Nicholson R. A. (1993) The dihydropyrazole RH-5529 blocks voltage-sensitive calcium channels in mammalian synaptosomes. *Pestic. Biochem. Physiol.* **45**, 242-247.
- Zheng J., Leong D., Lees G., and Nicholson R. A. (1998). Studies on the interaction of surangin B with insect mitochondria, insect synaptosomes and rat cortical neurons in primary culture. *Pestic. Biochem. Physiol.* **61**, 1-13.
- Zheng Y. J., Shapiro R., Marshall W. J. and Jordan D. B. (2000). Synthesis and structural analysis of the active enantiomer of famoxadone, a potent inhibitor of cytochrome *bcl*. *Bioorg. Med. Chem. Lett.* **10**, 1059-1062.
- Zimanyi I., Weiss S. R., Lajtha A., Post R. M. and Reith M. E. (1989). Evidence for a common site of action of lidocaine and carbamazepine in voltage-dependent sodium channels. *Eur. J. Pharmacol.* **167**, 419-22.
- Zimmermann H. (1993). *Synaptic transmission: Cellular and Molecular Basis.* (H. Zimmermann, Ed.). Oxford University Press, New York 31-67